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GRANT NUMBER DAMD17-97-1-7008

TITLE: Superoxide and Nitric Oxide Mechanisms in Traumatic Brain Injury and Hemorrhagic Hypotension

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REPORT DATE: December 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 1997	Annual (1 Dec 96 - 30 Nov 97)

4. TITLE AND SUBTITLE
Superoxide and Nitric Oxide Mechanisms in Traumatic Brain Injury and Hemorrhagic Hypotension

5. FUNDING NUMBERS
DAMD17-97-1-7008

6. AUTHOR(S)
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University of Texas Medical Branch
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200* Traumatic brain injury (TBI) reduces cerebral blood flow (CBF) and renders the brain vulnerable to secondary ischemia. Hypotension contributes to poor outcome after TBI in humans. We have prevented hypoperfusion and restored autoregulation after TBI. The goals of this project are to determine whether treatment based on our observations will prevent CBF reductions, brain edema and histological damage after TBI and hemorrhagic hypotension, and to understand the mechanisms that contribute to the efficacy of the proposed treatments. Specific Aim 1 addressed the hypothesis that impairment of cerebrovascular function will result in brain injury after TBI and hemorrhagic hypotension that would not occur after hypotension alone. Specific Aim 2 addressed the hypothesis that post-TBI cerebral hypoperfusion is caused by nitric oxide (NO)-dependent mechanisms. Specific Aim 3 addressed the hypothesis that increased production of superoxide during TBI and subsequent hypotension/resuscitation is responsible for the impaired cerebrovascular reactivity. Specific Aim 4 will address the hypothesis that small-volume resuscitation with hypertonic saline will restore cerebral circulatory and systemic hemodynamics without causing the pronounced changes in brain water diffusion seen after TBI and hypotension/resuscitation with shed blood.

14. SUBJECT TERMS	15. NUMBER OF PAGES
cerebral blood flow, free radicals, head trauma, hemorrhage, hypotension, nitric oxide, rat, shock	75
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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TABLE OF CONTENTS

INTRODUCTION:

Traumatic brain injury (TBI) reduces cerebral blood flow (CBF) and renders the brain vulnerable to secondary ischemia. There is clinical evidence that hypotension contributes to poor outcome after TBI, likely because of traumatic damage to the cerebral circulation. Our research has resulted in novel and important observations related to the mechanisms of traumatic vascular injury. During the past year we have observed that posttraumatic hypoperfusion can be prevented using substrates of nitric oxide synthase (NOS). We have also observed that NOS enzyme activity is not affected by our model of TBI, suggesting that TBI is affecting NO directly. We have observed that prolonged (over 45 minutes) superoxide anion radical production occurs after TBI and that posttraumatic hypoperfusion can be significantly reduced using oxygen radical scavengers. In addition, we have observed that while agonist-induced vasodilation and vasoconstriction are intact in rat cerebral arteries after TBI, myogenic responses to hypotension are significantly reduced by trauma. These exciting observations, described in detail below, are consistent with the overall goal of this project: to determine whether a treatment strategy based on our observations will prevent CBF reductions, brain edema and histological damage after TBI and hemorrhagic hypotension, as well as to understand the mechanisms that contribute to the efficacy of the proposed treatments.

BODY:

This section summarizes progress made in the previous year as related to each **Specific Aim**.

Specific Aim 1 is to address the hypothesis that impairment of cerebrovascular function will result in brain injury after TBI and hemorrhagic hypotension that would not occur after hypotension alone. We will relate changes in CBF and magnetic resonance imaging (MRI) evidence of brain edema to histopathological damage in rats with and without TBI and hypotension/resuscitation.

T2 spin-echo (SE) and diffusion-weighted imaging (DWI) during TBI and hypotension.

We adapted the fluid-percussion TBI model for application within the MRI unit in order to obtain serial pre- and post-TBI images. Application of these multimodal MRI techniques to the study of TBI provided new information on the consequences of decreased CBF resulting from hypotension after TBI. We demonstrated a decrease in the height of the arterial input function, dramatic intracerebral hemodynamic alterations and perfusion values consistent with the reduced CBF that we and other have observed using microspheres and laser Doppler flowmetry (LDF).

Proton MRI was performed with a 4.7-Tesla imaging system (SISCO, Fremont, CA) using a home-built 2-inch saddle-style radio frequency coil tuned to 200 MH. The rat was positioned in the magnet in a Plexiglas holder, on a circulating heated-water bath, and regulated hot air was blown through the magnet bore to maintain rectal temperature between 37°C and 38°C. Twelve SE and diffusion-weighted images were obtained, and imaging of plasma volume and transit time was performed using the intravascular tracers, ultrasmall superparamagnetic iron

oxide (USPIO) (AMI-227, Advanced Magnetics, Cambridge, MA) or dysprosium-DTPA (diethylene triaminepentaacetic acid), chelated in our laboratory by standard techniques and FLASH (fast low-angle shot) methods (10).

Changes in the integral of the arterial transit curves were used to represent relative changes in brain cerebral vascular resistance (CVR) during each experimental condition (see below). In several animals, injection of dysprosium-DTPA was followed by injection of USPIOs to assess the permeability of the blood-brain barrier (BBB) to the smaller dysprosium compound. The general experimental paradigm was either sham or fluid-percussion injury (FPI), and was followed by graded hemorrhagic hypotension followed by reperfusion. Measurements were made at baseline and after each condition. We extended the more conventional intracerebral determination of the transit of magnetic resonance contrast agent to the determination of transit curves in intracerebral and extracerebral arteries. We proposed that by assessing the volume of contrast agent in the cerebral arteries, relative changes in CVR could be determined, i.e., an increase in CVR relative to other vascular beds would be reflected in decreased volume of contrast agent in the internal carotid arteries. The proportion of contrast agent could be measured by integrating the arterial bolus transit curve. By combining the arterial input information with intraparenchymal transit curves, hemodynamic compensatory mechanisms could be quantified.

To test this hypothesis, we confirmed that the area under the curve (AUC) reflected the amount of contrast agent within the artery by injecting increasing doses of dysprosium-DTPA, and we found a linear relationship between dose injected and AUC ($n=4$, $r^2=.98$). Next, we studied transit curves in an intracerebral artery and in the brain parenchyma during various degrees of hemorrhagic hypotension. Blood vessels in the circle of Willis were identified based on characteristic location, hyperintensity at baseline in the gradient-echo (GRE) movie, and demonstrating the earliest arrival of the bolus. In uninjured rats during controlled hemorrhage, blood volume increased progressively as blood pressure was decreased to a mean arterial pressure (MAP) of 40 mmHg, at which point the volume decreased, consistent with a passive reduction in flow when the lower level of autoregulation was breached (Table 1). Concurrently, dramatic increases in intraparenchymal contrast volume were obtained as blood pressure was lowered to 60 mmHg, consistent with compensatory mechanisms to maintain CBF (Table 1). Like the arterial input function, intraparenchymal blood volume also decreased at MAP 40 mm Hg, consistent with loss of autoregulation.

Table 1. Arterial and brain parenchymal transit curves in an uninjured rat.

Blood Pressure (mm Hg)	Intracranial Artery		Brain	
	First Moment[1]	Blood Volume[2]	First Moment	Blood Volume
110	3.45	1575	3.49	954
80	4.77	3454	6.78	2375
60	5.96	4691	6.15	2360

40	3.96	2659	4.56	1721
100	4.12	2023	4.53	1416

[1] transit time (seconds) [2] integral of transit curve

In contrast, after moderate (2.2 atm) TBI, a 60 ± 30% (mean ± S.D.) decrease in contrast enhancement in the arterial input was observed (n=4). Calculation of CBF within the brain parenchyma demonstrated that TBI alone reduced CBF by 50%, with a further decrease in CBF during hypotension (Table 2). Arterial transit time decreased, suggesting vasoconstriction in large branches of the cerebral arterial circle (of Willis) such as the anterior and middle cerebral arteries. Moreover, an increase in the area under the tracer transit curve occurred within the brain, suggestive of small vessel dilation, such as might occur downstream from constricted larger vessels. These results support the hypothesis that TBI results in an increase in cerebral arterial tone, which alters the baseline for subsequent hypotensive challenges. After hemorrhagic hypotension, some capacity for vasodilation remains, but is insufficient to compensate for the reduction in perfusion pressure. Thus, the events underlying loss of autoregulation after TBI are quite different from what occurs in an uninjured animal, in which loss of autoregulation occurs only after maximal vasodilation can no longer sufficiently compensate for reduction in perfusion pressure.

Table 2. Effect of traumatic brain injury (TBI) and hypotension on cerebral hemodynamics

Mean arterial blood pressure levels	Sham TBI[1]			TBI (2.2 atm.)		
	corMTT[2]	CBV	Flow	corMTT[2]	CBV	Flow
Normotension	100	100	100	260	163	64
Hypo. (60 mmHg)	405	382	94	680	290	42

[1]all values are percent of normotensive, untraumatized control animals

[2]corMTT = mean transit time corrected for time of arrival and first moment of the arterial input function

CBV, cerebral blood volume; Hypo, hypotension.

Effects of TBI and hypotension on apparent diffusion coefficient (ADC).

To address the hypothesis that TBI followed by hypotension and resuscitation causes water shifts within the brain, we determined the effect of TBI and hemorrhagic hypotension/resuscitation on ADC. The ADC was quite stable in sham animals with and without hemorrhagic hypotension/resuscitation (Table 3). TBI alone reduced the ADC by less than 10%, with no change seen with subsequent reduction of MAP to 60 mmHg. However, a dramatic reduction in ADC was seen after resuscitation, suggesting that alterations in cellular membrane properties occurred with resuscitation. ADC maps revealed that the most dramatic reduction in ADC occurred in white matter and hippocampal regions.

Table 3. Apparent diffusion coefficient (ADC) values ($mm^2/sec \cdot 10^{-3}$)

Measurement interval	Sham-TBI	Moderate TBI
Pre-TBI baseline	549	552
TBI/sham TBI	538	504
Hypotension (60mmHg)	557	528
Reinfusion	558	308

TBI, traumatic brain injury.

The decreases in ADC we observed after reinfusion might indicate free-radical-mediated reperfusion injury. An involvement of O_2^- in reperfusion injury is supported by our preliminary results measuring O_2^- using the cytochrome C-coated platinum electrode implanted on the brain surface during TBI and hypotension (8).

Histological studies of TBI and hemorrhagic hypotension.

To determine whether TBI followed by hemorrhagic hypotension would produce histological changes not produced by TBI alone, rats were anesthetized with 1.4% isoflurane and prepared for TBI. After moderate (2.2 atm) TBI, rats were hemorrhaged via a cannula in a femoral artery to a MAP of 60 mmHg for 45 min, after which time shed blood was reinfused and rats were allowed to survive for 4 hr. Rats were randomly assigned to 1 of 4 groups (sham injury with no hypotension, sham injury with hypotension, TBI without hypotension and TBI plus hypotension, n=4 per group). All rats were then perfused transcardially with warmed 0.9% saline followed by 10% neutral buffered formalin. Brain were cut in 5-mm coronal sections, dehydrated, cleared with graded alcohols, and infiltrated with paraffin. Coronal sections (8 μm) were cut and stained with hematoxylin and eosin (H&E) and examined, with a focus on hippocampus and on arterial boundary zones. Histologic preparation and neuropathological examination were performed by a neuropathologist who was blinded to the experimental group (Marjorie R. Grafe, M.D., Ph.D., Department of Pathology, UTMB). Although there was evidence of ischemic neuronal injury (dark neurons, swollen perineuronal spaces) in the TBI plus hypotension group, it did not consistently occur at cerebral arterial boundary zones in all rats in that group. We are investigating longer periods and lower levels of hypotension with longer survival times to determine whether hypotension after TBI produces more ischemic neuronal injury than hypotension in the absence of TBI.

Specific Aim 2 is to address the hypothesis that post-TBI cerebral hypoperfusion is caused by nitric oxide (NO)-dependent mechanisms. NO synthase (NOS) activity will be assessed post-TBI to determine its contribution to the vascular effects observed.

CBF after fluid-percussion TBI in rats: treatment with L-arginine and superoxide dismutase (SOD).

Rats anesthetized with isoflurane (1.5%) were prepared for moderate fluid-percussion

TBI and randomly assigned to receive a saline placebo (n=8) , L-arginine (100 mg/kg, i.v., 5 min post-TBI, n=8), SOD (24,000 U/kg, i.v. bolus before TBI plus 1,600 U/kg/min for 10 min starting immediately after TBI, n=8), L-arginine plus SOD (n=8) or sham-injury plus L-arginine (n=8). CBF was measured (by LDF) for 2 hours after TBI. In the rats treated with saline placebo, CBF decreased significantly after TBI (Figure 1), confirming previous microsphere (12-14) and LDF (11) reports of transient hypoperfusion that occurs after fluid-percussion TBI in rats. Rats treated with L-arginine without injury exhibited no change in CBF. The rats treated with L-arginine exhibited no CBF decreases and the SOD-treated rats exhibited CBF decreases that resolved within 45 min of TBI (6). A subsequent group of rats (n=5 per group) was prepared identically but received a saline placebo, L-arginine (100 mg/kg, i.v., 5 min post-TBI) or D-arginine (100 mg/kg, i.v., 5 min post-TBI). CBF decreased significantly in the groups treated with the placebo or D-arginine but did not change in the L-arginine treated group (Figure 2).

These observations suggest that posttraumatic hypoperfusion is caused by NO destruction by $O_2^{\cdot-}$.

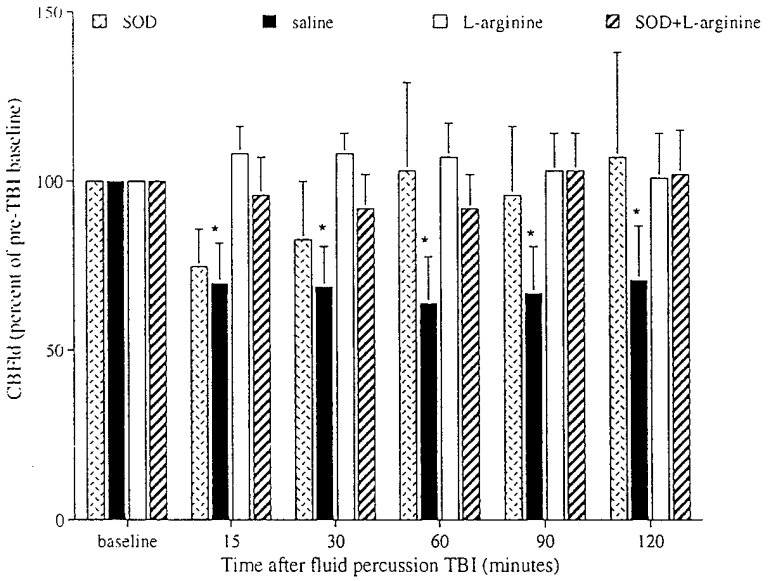

Figure 1. Cerebral blood flow (laser Doppler flowmetry) after moderate (2.2 atm) TBI in rats treated with SOD, saline placebo, L-arginine or SOD + L-arginine.

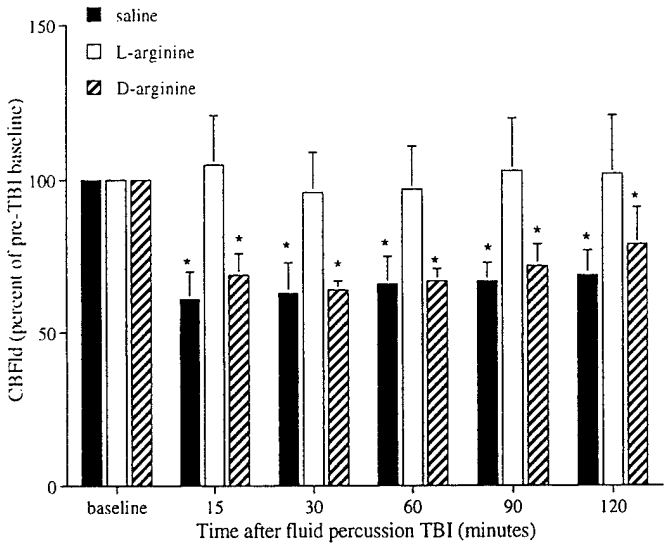

Figure 2. Cerebral blood flow (laser Doppler flowmetry) after moderate (2.2 atm) TBI in rats treated with L-arginine, saline placebo, or D-arginine.

Effects of NOS inhibition on CBF and cerebral vascular reactivity to hypotension and hemodilution.

To test the hypothesis that TBI-induced destruction of NO may contribute to impaired cerebral vascular reactivity we investigated whether NO was the primary mediator of cerebral vasodilatory responses to hypotension or the cerebral hyperemic response to hemodilution. We measured the effects of the NOS inhibitors L-NMMA (NG-monomethyl-L-arginine, 25 mg/kg, n=8) and L-NAME (NG-nitro-L-arginine methyl ester, 50 mg/kg/hr) on resting CBF and on CBF responses to hypotension and hemodilution. In rats anesthetized with 1.5% isoflurane, microsphere CBF measurements were performed. CBF was determined either before and 15 and 30 minutes after (n=7) or 30, 60 and 90 minutes (n=8) after the beginning of a continuous infusion of L-NAME (50 mg/kg/hr). In an additional group of rats (n=6), CBF was measured continuously using LDF during a continuous infusion of L-NAME (50 mg/kg/hr, i.v.). In order to test the effects of NOS inhibition on autoregulation, rats were treated with saline (n=6), L-NMMA (25 mg/kg, n=6) or L-NAME (25 mg/kg, n=6). CBF was measured before and after MAP was lowered to levels of 80 and 60 mmHg. The effect of NOS inhibition on hyperemic responses to hemodilution was tested in rats treated with saline (n=6) or L-NAME (25 mg/kg, n=6), and CBF was measured before and after 30% and 60% of blood volume was exchanged for 0.9% saline. In the L-NAME infusion groups, CBF (both microsphere and LDF) decreased ($p < 0.05$, CBF-LDF vs baseline) after 30 minutes of L-NAME infusion and remained constant for the next 60 min (Figure 3). CBF remained constant in all three groups as MAP was decreased, indicating that autoregulation was unaffected by NOS inhibition (Figure 4). CBF increased 49% and 68% in the saline and L-NAME-treated groups, respectively, after isovolemic

replacement of 60% of total blood volume (Figure 5), indicating that hyperemic responses to hemodilution were intact after NOS inhibition. Our data indicate that inhibition of NOS decreases resting CBF but does not impair autoregulation or prevent compensatory increases in CBF that occur after isovolemic hemodilution. Although these data indicate that traumatic effects on cerebral vasodilatory responses to hypotension are probably not mediated primarily through NO, the role of NO in impaired cerebral vascular reactivity after TBI will be investigated further (see below).

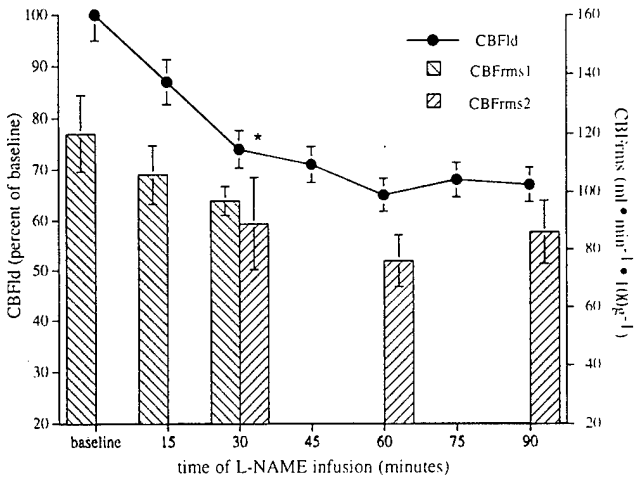

Figure 3. Cerebral blood flow in rats treated with a continuous infusion of L-NAME (NG-nitro-L-arginine methyl ester, 50 mg/kg/hr). Bars indicate microsphere CBF measurements (right vertical axis). Line indicates that laser Doppler CBF values are a percent of pre-L-NAME baseline (left vertical axis). * $p < 0.05$ vs. pre-L-NAME baseline.

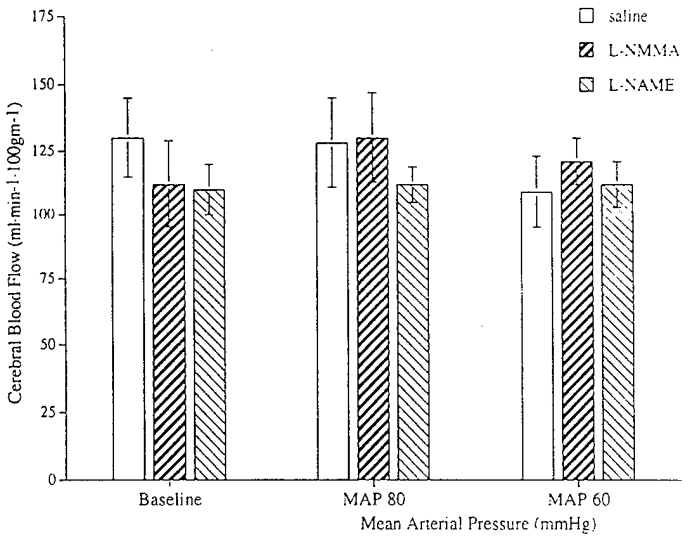

Figure 4. Cerebral blood flow at baseline (15 min after treatment) and after hemorrhage to mean arterial pressure levels of 80 mmHg and 60 mmHg in rats treated with saline (n=6), L-NMMA (NG-monomethyl-L-arginine, 25 mg/kg, n=8), or L-NAME (25 mg/kg, n=6).

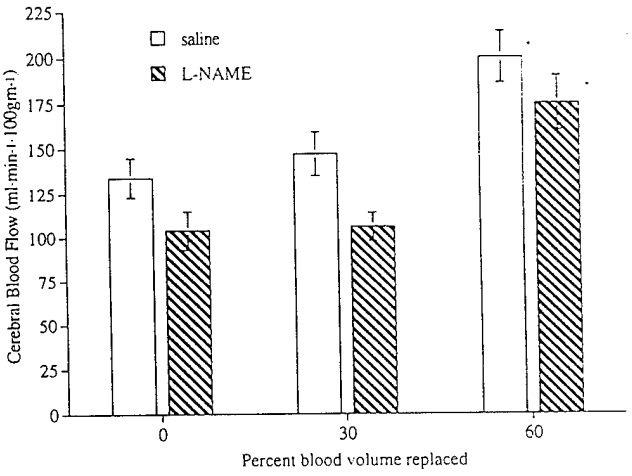

Figure 5. Cerebral blood flow at normodilution (0) 15 min after treatment with L-NAME, 25 mg/kg (n=8), or saline (n=6) and after replacement of 30% and 60% of blood volume was exchanged for isotonic saline. *significantly ($p<0.05$) different from baseline (0).

Effects of NOS inhibition on CBF after TBI.

Recently, we reported that L-arginine, but not D-arginine, improves CBF after TBI in rats, suggesting that TBI reduces CBF by NO-related mechanisms. If TBI decreases CBF by reducing NO levels then inhibition of NO synthesis should reduce CBF to levels similar to those observed after TBI. In addition, if TBI is inactivating NO, then NOS inhibition after TBI should not produce additional CBF decreases. To test these hypotheses, rats were treated with the NOS inhibitor L-NAME after TBI or sham-TBI. In a fourth group mean arterial pressure was maintained constant during L-NAME infusion using an aortic reservoir. Experiments were approved by the Animal Care and Use Committee of the University of Texas Medical Branch. Male Sprague-Dawley rats (400-450g) were anesthetized with 1.5-2% isoflurane in O_2:air (70:30), intubated, and ventilated before surgical preparation for midline fluid-percussion TBI as described (7). Briefly, the rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4.8-mm hole was trephined into the skull over the sagittal suture and a modified LuerLok syringe hub was place over the exposed dura and bonded in place with cyanoacrylic and dental cement. In one group a polyethylene catheter (PE 205) was placed in the abdominal aorta and connected to a reservoir that could be raised or lowered to maintain arterial blood pressure, which was monitored via a polyethylene catheter in one femoral artery.

After surgery, isoflurane was lowered to 1.0-1.5%; the rats were connected to the trauma device, and subjected to moderate (2.2 atm.) TBI or sham-TBI. They were then randomly assigned to receive moderate TBI alone (TBI, n=5), sham-TBI followed 30 minutes later by a bolus of L-NAME (Sham+LNAME, 30mg/kg, i.v., n=5), moderate TBI followed 30 minutes later by a bolus of L-NAME (TBI+LNAME, 30mg/kg, i.v., n=5), moderate TBI followed 30 minutes later by a bolus of L-NAME with controlled mean arterial blood pressure to prevent arterial hypertension during L-NAME infusion (TBI+NAME+AB, 30mg/kg, i.v., n=5). In all groups, arterial blood pressure and CBF were measured continuously for 120 min after L-NAME infusion. NOS inhibition and moderate TBI reduced CBF to similar degrees. After TBI, CBF returned towards baseline within 2 hours while CBF decreases due to NOS inhibition persisted for the 120-min measuring period (Figure 6). The combination of TBI and L-NAME reduces CBF more than either treatment alone. Although the observation that L-NAME and TBI reduce CBF to similar levels suggests that both reduce NO to a similar degree, the observation that NOS inhibition further reduces CBF suggests that some NO-mediated vasodilation remains after TBI. Further studies with more complete NOS inhibition, preferably with specific NOS inhibitors, are required to more accurately address the role of NO in posttraumatic hypoperfusion.

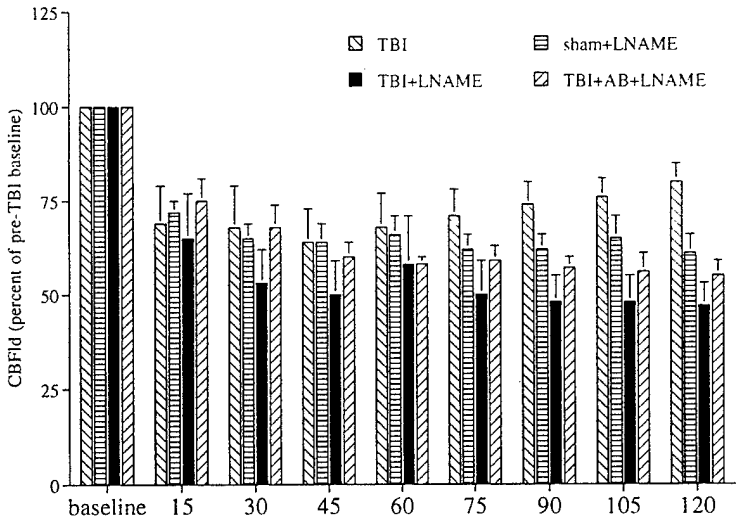

Figure 6. Cerebral blood flow (laser Doppler flowmetry) moderate (2.2 atm) TBI in rats treated with a saline placebo (TBI), sham-TBI plus L-NAME (Sham+LNAME), TBI plus L-NAME (TBI+LNAME), TBI followed 30 minutes later by L-NAME with controlled arterial blood pressure (TBI+NAME+AB).

NOS activity after TBI.

To determine whether TBI would alter total NOS activity, assays of the conversion of arginine to citrulline were made in crude enzyme preparations, as described by Bredt and Snyder (3). Briefly, rats were decapitated and their brains removed and homogenized. The homogenate was centrifuged for 5 min at 3000 · g and the supernatant was spun at 20,000 · g for 15 min. The supernatant from the second spin was passed over an ion exchange column to remove endogenous arginine. Activity of the NOS was monitored by measuring the levels of [3H]citrulline. Measurable NOS activity exists in preliminary experiments without the addition of exogenous tetrahydrobiopterin; thus, this cofactor was not routinely added. These data are presented as amount of [3H]citrulline divided by the amount of [3H]arginine + [3H]citrulline · 100 / mg of protein.

There were no significant differences in NOS activity among control (n=10, unoperated), sham (n=5, prepared for TBI but not injured) or injured [n=5, moderate (2.2 atm) fluid-percussion TBI] rats at either low (0.45 μM) or high (1.50 μM) added L-arginine levels (Figure 7).

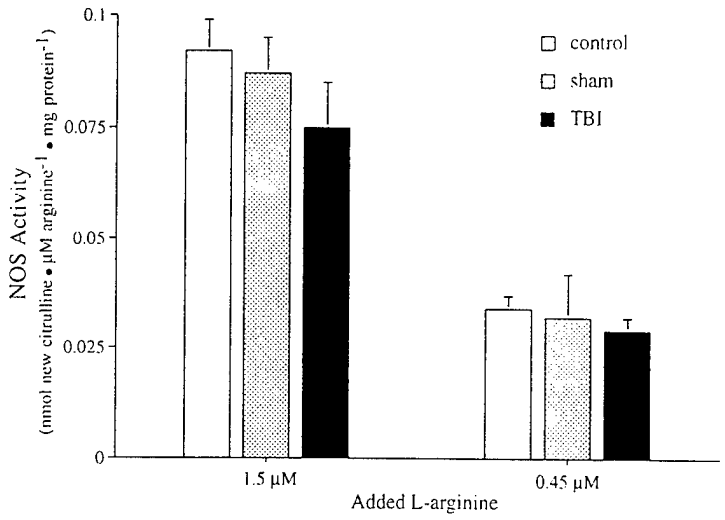

Figure 7. Total baseline soluble nitric oxide synthase (NOS) activity determined from crude enzyme preparations from control (unoperated, n=10) rats and rats subjected to sham injury (n=5) or moderate (n=5) fluid-percussion TBI.

In order to determine whether stimulated NOS activity was affected by TBI, NOS activity was measured in minced tissue preparations using our modification (1) of previously described methods (3). After moderate TBI, rats were decapitated and the frontal cortices were dissected, cross-chopped and minced into slices (450 x 450 μm). The conversion of L-[3H]-arginine to L-[3H]-citrulline in the cortical slices was used as an index of NOS activity. The methods of Bredt and Snyder (3) were modified to the extent that we used a 5-min stimulation period. NOS activity in response to stimulation with NMDA (μM), a calcium ionophore (inomycin, 10 μM) or K^+ (50 mM) was determined in injured (2.2 atm TBI) or sham-injured rats.

There was no difference in NOS activity in response to stimulation with NMDA (300 μM), K+ (50 mM) or ION (inomycin,10 μM) in control rats (n=9, no surgery) or in rats after moderate TBI (n=5, 2.2 atm) or sham-injury (n=5, preparation for TBI but no injury) (Figure 8).

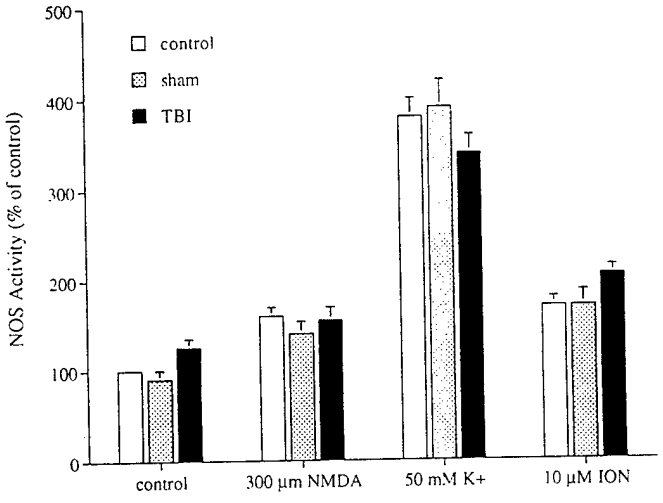

Figure 8. Stimulated cell-dependent NOS activity in mince preparations from control (unoperated, n=9) rats and rats subjected to sham injury (n=5) or moderate fluid-percussion TBI (n=5) and then exposed to N-methyl-D-aspartate (NMDA), potassium chloride (K^+), or ionomycin (ION).

Specific Aim 3 is to address the hypothesis that increased production of O_2^- during TBI and subsequent hypotension/resuscitation is responsible for the impaired cerebrovascular reactivity.

Superoxide radical production and CBF after TBI.

CBF and O_2^- production were measured in rats treated with an experimental free radical scavenger and inhibitor of lipid peroxidation (U-74389G, 16-desmethyl-tirilazad, Upjohn/Pharmacia) to determine whether changes in O_2^- and CBF were related. Using a cytochrome C-coated platinum electrode, we measured O_2^- production in isoflurane-anesthetized rats after moderate TBI (8). Superoxide levels increased after TBI and local and systemic injection of SOD transiently reduced O_2^- level. In a second series of rats, CBF and O_2^- levels were measured simultaneously. Cytochrome C electrodes were constructed of platinized carbon electrode (PACE) material and calibrated for sensitivity to O_2^- using the xanthine-xanthine oxidase system. Rats (n=12) were anesthetized with isoflurane, intubated, mechanically ventilated with 1.5-2.0% isoflurane and prepared for moderate fluid-percussion TBI (2.2 atm); recordings of O_2^- production were made using an electrode positioned 2 mm lateral to the midline and anterior to bregma using a DCV-5 potentiostat (E'-Chem, BAS). A counter-electrode was positioned contralaterally and the reference electrode was attached to the skin. Recordings were made with an applied voltage of 0.05mV relative to a saturated mercury-

calamine electrode. Relative CBF was measured using a laser Doppler flow probe placed over the thinned calvaria contralateral to the injury site. After surgical preparation, isoflurane was lowered to 1.5% and CBF was monitored continuously and expressed as mean ± SEM percent of preinjury perfusion. Rats were treated with either U-74389G (3mg/kg, n=6) or a carrier (20 mM sodium citrate, 77 mM NaCl, n=6) subjected to moderate fluid-percussion TBI. An additional group of rats (n=3) was prepared identically but the PACE electrode was coated with bovine serum albumin instead of cytochrome C. CBF and O_2^- production were measured for 90 min after TBI.

After TBI in both the U-74389G and carrier-treated groups, CBF decreased and remained below baseline for the 90-min measurement period (Figure 9). In the carrier-treated group, O_2^- levels rose to a peak at about 30 min after TBI (Figure 10, open circles) and then remained elevated for the 90 min measurement period. O_2^- rose in the U-74389G-treated group after TBI but to significantly lower levels and then returned to baseline levels within 80 min of TBI (Figure 10, closed circles). There was no change in signal after TBI in the group with the electrode that was not coated with cytochrome C (Figure 10, closed triangles). Interestingly, there was a disassociation between O_2^- levels, which decreased in the U-74389 group, and CBF, which was not affected by treatment. These observations suggest that either increases in O_2^- do not contribute to posttraumatic hypoperfusion or that the increases in O_2^- that occurred after TBI in the U-74389G-treated rats were sufficient to inactivate NO and produce cerebral hypoperfusion.

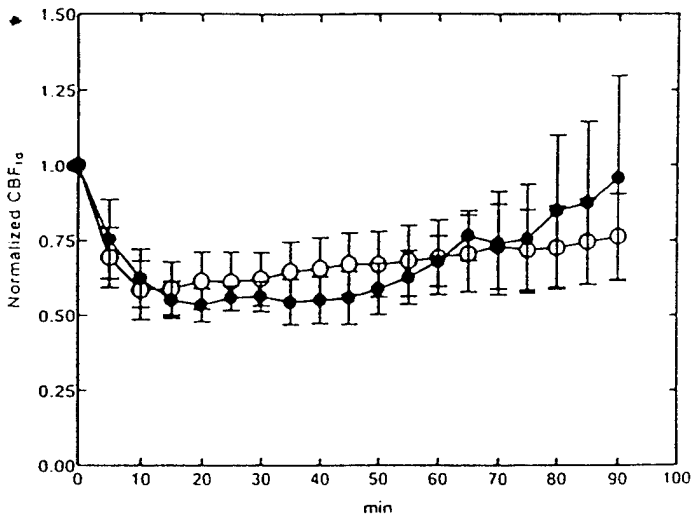

Figure 9. Cerebral blood flow (laser Doppler flowmetry) measurements after moderate (2.2 atm) TBI in rats treated with a carrier (closed circles, n=6) or with U-74398G (open circles, n=6). CBF LD measurements are percent of pre-TBI baseline.

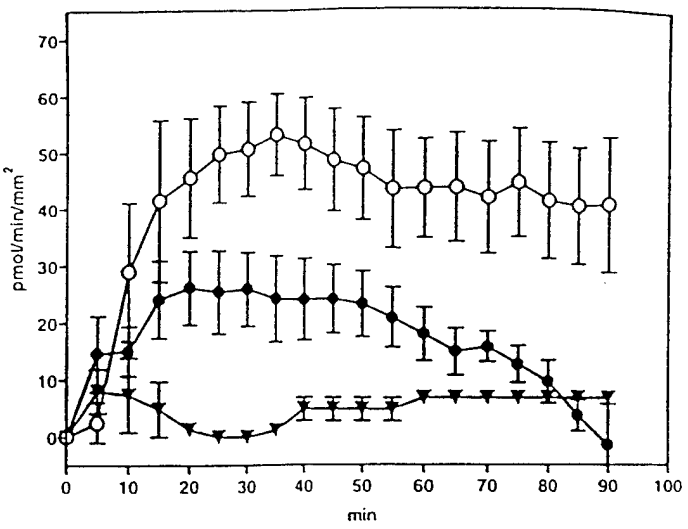

Figure 10. O_2^- measurements after moderate (2.2 atm) TBI in rats treated with a carrier (closed circles, n=6) or with U-74398G (open circles, n=6). An additional group of rats (n=3) was prepared identically but the PACE electrode was coated with bovine serum albumin instead of cytochrome C (closed triangles).

CBF responses to controlled hemorrhagic hypotension (autoregulation) after TBI: effects of SOD treatment.

Using LDF in isoflurane-anesthetized rats, CBF was recorded during periods of hemorrhagic hypotension before and after TBI. Hypotension was produced by bleeding into a reservoir connected to the abdominal aorta via a polyethylene cannula placed distal to the renal arteries. Raising or lowering the reservoir produced steady changes in MAP in 10-mmHg increments. An autoregulatory challenge was performed before injury and then at 30-min intervals for 120 min after TBI. In untreated rats (n=4), autoregulation was impaired after TBI, as demonstrated by greater decreases in CBF during hypotension after TBI than before TBI. Impaired autoregulation persisted for 120 min after TBI. In rats treated with SOD [24,000 U/kg, i.v. bolus before TBI plus 1,600 U/kg/min for 10 min starting immediately after TBI (n=5)], CBF during hypotension was improved at 30 min post-TBI (Figure 11) and was restored to pre-TBI levels at 90 min post-TBI (Figure 12).

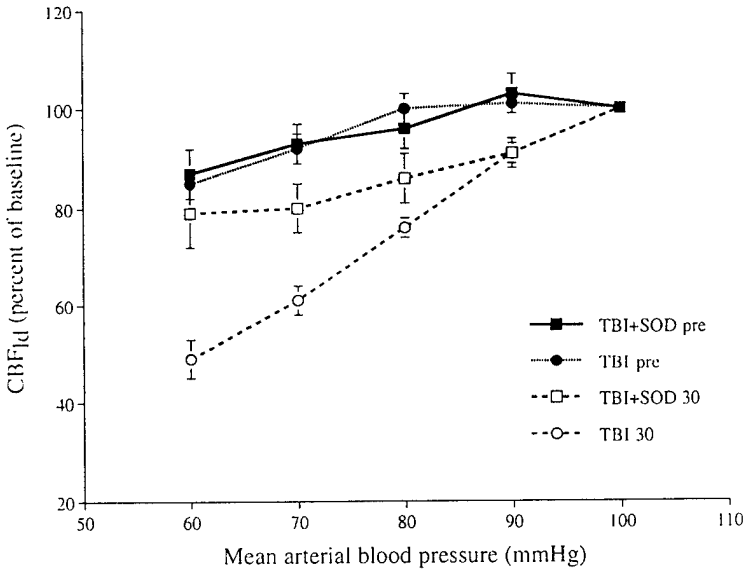

Figure 11. Cerebral blood flow (laser Doppler flowmetry) during hemorrhagic hypotension 30 min after moderate fluid-percussion injury (2.2 atm) in rats untreated (n=4) or treated with SOD (n=5).

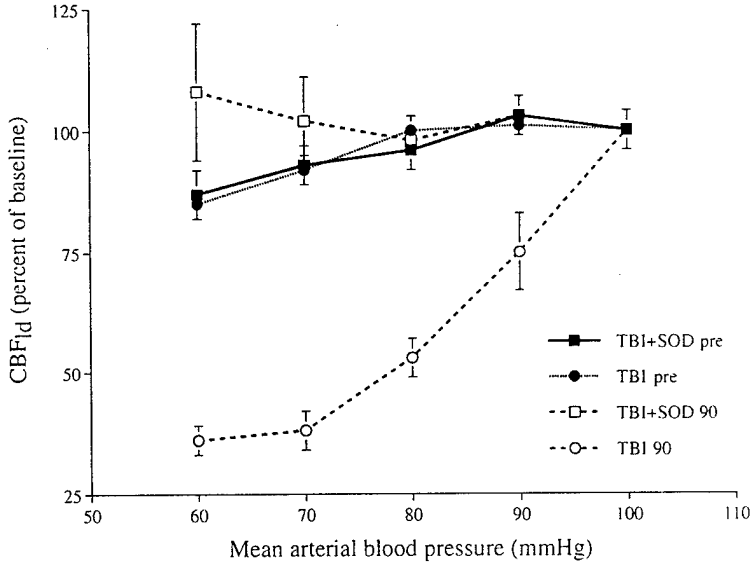

Figure 12. Cerebral blood flow (laser Doppler flowmetry) during hemorrhagic hypotension 90 min after moderate fluid-percussion injury (2.2 atm) in rats untreated (n=4) or treated with SOD

(n=5).

Specific Aim 4 will address the hypothesis that small-volume resuscitation with hypertonic saline will restore cerebral circulatory and systemic hemodynamics without causing the pronounced changes in brain water diffusion seen after TBI and hypotension/resuscitation with shed blood.

The objectives of Specific Aim 4 will be addressed in the second and third years of the funding period.

CONCLUSIONS

These studies demonstrate that significant progress towards the aim of the application have been made in the first year. Our research has resulted in novel and important observations related to the mechanisms of traumatic vascular injury. We have reported that TBI results in transient hypoperfusion (14) and in regionally specific impairment or abolition of pressure autoregulation (4;5). We have also observed that L-arginine and SOD prevent or reverse cerebral hypoperfusion (6), suggesting a free-radical-mediated reduction in NO or NOS activity after TBI. NOS activity is not influenced by fluid-percussion TBI, suggesting that TBI may destroy NO directly rather than reducing NOS activity. We have reported significant increases in O_2^- radical production after TBI (8;9). O_2^- can inactivate NO, perhaps by converting it to peroxynitrite (2). Our preliminary evidence suggests that cerebral autoregulatory responses to hemorrhagic hypotension can be restored by treatment with oxygen free radical scavengers. We have observed that NOS inhibitors further reduce CBF after TBI, suggesting that TBI destroys some, but not all, of the NO that contributes to normal vasodilatory tone in the cerebral circulation. These hypotheses will be investigated further during the remaining years of the funding period.

PUBLICATIONS

The following articles, book chapter, editorials and abstracts were published or submitted during the previous funding period. Items included in Appendix 1 are marked with an asterisk.

*1. Mottet, I, Quast MJ, DeWitt DS, Hillman GR, Wei J, Uhrbrock DH, Perez-Polo JR, Kent TA. Nω-nitro-L-arginine methyl ester modifies the input function measured by dynamic susceptibility contrast MRI. J Cereb Blood Flow Metab 17:791-800, 1997

*2. DeWitt DS, Smith TG, Deyo DS, Miller KR, Uchida T, Prough DS. L-arginine or superoxide dismutase prevents or reverses cerebral hypoperfusion after fluid-percussion traumatic brain injury. J Neurotrauma 14:223-233, 1997

3. Prough DS, DeWitt DS, Jenkins LW. Experimental traumatic brain injury: implications for clinical treatment. In: Neuroanesthesia, eds. Johnson JO, Sperry RJ, Stanley TH. Kluwer Academic Publishers, Netherlands, 123-138, 1997

4. Prough DS, DeWitt DS, Jenkins LW. Secondary injury after clinical traumatic brain injury. In: Neuroanesthesia, eds. Johnson JO, Sperry RJ, Stanley TH. Kluwer Academic Publishers, Netherlands, 153-164, 1997

*5. Bukoski RD, Wang SN, Bian K, DeWitt DS. Traumatic brain injury does not alter cerebral contractility. Am J Physiol 272:H1406-H1411, 1997

6. DeWitt DS. Guest editorial response to Puurunen, et al., Spatial learning after global ischemia. Stroke 28:631, 1997

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8. DeWitt DS, Alagarsamy S, Johnson KM, Prough DS. Traumatic brain injury does not reduce total or stimulated nitric oxide synthase activity. J Cereb Blood Flow Metab 17(Suppl 1): S17, 1997 (abstract)

9. Kent TA, Mottett I, Quast MJ, DeWitt DS, Hillman GR. Quantitative cerebral blood flow changes using contrast enhanced MRI: modulation by nitric oxide. J Cereb Blood Flow Metab 17(Suppl 1): S14, 1997 (abstract)

*10. DeWitt DS, Prough DS, Uchida T, Deal DD, Vines SM. The effects of nalmefene, CG3703, tirilazad or dopamine in cerebral blood flow, oxygen delivery, and electroencephalographic activity after traumatic brain injury and hemorrhage. J Neurotrauma 14:931-941, 1997

11. DeWitt DS, Tidwell CW, Prough DS. Cerebral hypoperfusion after brain injury due to nitric oxide (NO) and non-NO related mechanisms. Ann Thorac Surg (in press), 1997 (abstract)

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Neurotrauma (revised and resubmitted).

*16. Alagarsamy S, DeWitt DS, Johnson KM. Traumatic brain injury does not affect nitric oxide synthase activity in rats. J Neurotrauma (revised and resubmitted).

17. Bedell, DeWitt DS, Prough DS. Fentanyl infusion preserves cerebral blood flow during decreased mean arterial blood pressure following traumatic brain injury in cats. J Neurotrauma (in review).

18. DeWitt DS, Prough DS, Deal DD, Vines SM, Uchida T. Inhibition of nitric oxide synthase does not reduce cerebral vascular responses to hemodilution or hemorrhagic hypotension in rats. J. Neurosurg Anes (in review).

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APPENDIX 1

Appendix 1 includes copies of the following publications:

1. Mottet I, Quast MJ, DeWitt DS, Hillman GR, Wei J, Uhrbrock DH, Perez-Polo JR, Kent TA. Nω-nitro-L-arginine methyl ester modifies the input function measured by dynamic susceptibility contrast MRI. J Cereb Blood Flow Metab 17:791-800, 1997

2. DeWitt DS, Smith TG, Deyo DS, Miller KR, Uchida T, Prough DS. L-arginine or superoxide dismutase prevents or reverses cerebral hypoperfusion after fluid-percussion traumatic brain injury. J Neurotrauma 14:223-233, 1997

3. Bukoski RD, Wang SN, Bian K, DeWitt DS. Traumatic brain injury does not alter cerebral contractility. Am J Physiol 272:H1406-H1411, 1997

4. DeWitt DS, Prough DS, Uchida T, Deal DD, Vines SM. The effects of nalmefene, CG3703, tirilazad or dopamine in cerebral blood flow, oxygen delivery, and electroencephalographic activity after traumatic brain injury and hemorrhage. J Neurotrauma 14:931-941, 1997

5. Alagarsamy S, DeWitt DS, Johnson KM. Traumatic brain injury does not affect nitric oxide synthase activity in rats. J Neurotrauma (revised and resubmitted).

Journal of Cerebral Blood Flow and Metabolism
17:791–800 © 1997 The International Society of Cerebral Blood Flow and Metabolism
Published by Lippincott–Raven Publishers, Philadelphia

N^G-Nitro-L-Arginine Methyl Ester Modifies the Input Function Measured by Dynamic Susceptibility Contrast Magnetic Resonance Imaging

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Summary: In rat brain dynamic susceptibility contrast magnetic resonance (MR) images, vessels visible on the same scan plane as the brain tissue were used to measure the characteristics of the input function of the MR contrast agent gadopentetate dimeglumine. MR images were acquired 30 and 60 minutes after intravenous injections of 3 mg/kg and 15 mg/kg N^G-Nitro-L-arginine methyl ester (L-NAME) (n = 9). The time of arrival (TOA) and the mean transit time corrected for TOA of the input function were increased by 3 mg/kg or 15 mg/kg L-NAME. The area of the input function was increased by 15 mg/kg L-NAME. In two animals, similar modifications of the input function induced by 20 mg/kg L-NAME were reversed by infusion of sodium nitroprusside. In two other animals, MABP was increased by phenylephrine to a similar extent as in L-NAME experiments, but did not induce the same modifications of the input function, showing that the action of L-NAME on the input function was not simply caused by an effect on MABP. These results show that the input function can be significantly altered by manipulations widely used in cerebrovascular studies. These input function changes have important implications for calculation of cerebral blood flow. **Key Words:** Magnetic resonance imaging—Blood flow— Dynamics—brain perfusion—L-NAME.

Since the first descriptions of techniques allowing the assessment of hemodynamic parameters by using noninvasive T2*-sensitive nuclear magnetic resonance (NMR) bolus track measurements (Villringer et al., 1988; Majumdar et al., 1988; Belliveau et al., 1990; Moseley et al., 1991), this method has found widespread applications in the evaluation of neurological diseases and cerebrovascular perturbations. While passing through the microvascular bed, a bolus of paramagnetic contrast material such as gadopentetate dimeglumine alters the magnetic susceptibility of tissues and produces local field inhomogeneities, leading to reductions in T2 and T2* of the tissue. This results in transiently decreased signal intensities from T2*-weighted pulse sequences such as gradient-echoes. Acquiring a series of rapid T2*-weighted gradient-echo images during the first pass of the contrast agent bolus allows the reconstruction of a signal intensity-time curve that can be later converted into concentration-time curves. Using the indicator dilution theory, the concentration-time data can be used to calculate blood volumes and flows. In conditions where the blood-brain barrier is not impaired, gadopentetate dimeglumine remains intravascular in the brain, and the indicator dilution theory for intravascular tracers can be applied.

However, for calculation of CBF, this theory requires knowledge of the concentration-time curve of the tracer corresponding to the input of the observed tissue: the input function (Axel, 1980). Most of the previous magnetic resonance imaging (MRI) perfusion studies by dynamic susceptibility contrast experiments were per-

Received July 5, 1996; final revision received March 12, 1997; accepted March 12, 1997.

This work was funded by National Institutes of Health grants IRO1NS33288-01 and IRO1NS33575-01. Dr I. Mottet was supported by a grant from the Faculty of Medicine, Université Catholique de Louvain, Belgium.

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Abbreviations used: CBV, cerebral blood volume; CVR, cerebral vascular resistance; L-NAME, N_ω-Nitro-L-arginine methyl ester; MRI, magnetic resonance imaging; SNP, sodium nitroprusside; TOA, time of arrival.

formed in conditions where a constant input function could be assumed, such as the comparison of different areas of the same brain, allowing the calculation of relative values of cerebral blood volume (CBV) and CBF without measuring the input function.

The purpose of the present study was to determine whether changing physiological conditions would change the input function. In rat brain MR perfusion images, arteries and veins visible in the same scan plane as the brain tissue were used to measure the parameters of the input function. The physiological conditions were changed by injecting the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), already known to modify the resistance of cerebral arteries and CBF (Prado et al., 1992; Pelligrino et al., 1993). In two rats, the nitric oxide donor sodium nitroprusside (SNP) was given to reverse the modifications of the input function induced by 20 mg/kg L-NAME (Moncada et al., 1991; Smith et al., 1995). In two other rats, the MABP was increased by phenylephrine infusion to a similar extent as the hypertension induced by L-NAME injections, and the modifications of the input function compared with those of the L-NAME group. The implications of these input function changes were illustrated by evaluating CBV and CBF changes in the different conditions.

PRINCIPLES OF FLOW MEASUREMENT BY THE INDICATOR DILUTION THEORY

The indicator-dilution method using intravascular tracers converts the measurement of indicator concentration distal to an organ into flow and volume information about this organ (Zierler, 1965). This theory has been extended to the calculation of cerebral perfusion based on rapid sequence tomographies performed simultaneously with the bolus injection of a nondiffusible indicator (Axel, 1980): The Central Volume Theorem states that tissue blood flow (F) can be determined by the ratio

$$F = \frac{V}{MTT} \quad (1)$$

where V is the volume of distribution of the agent within the tissue (for an intravascular contrast agent, V is the tissue blood volume), and MTT is the mean transit time, or normalized first moment, of the outflow concentration-time curve $C_{out}(t)$ resulting from a bolus injection.

$$MTT = \frac{\int_0^\infty t\, C_{out}(t)dt}{\int_0^\infty C_{out}(t)dt} \quad (2)$$

Assuming that the concentration of the contrast material is the same at both points, $C_{out}(t)$ may be measured either at the outflow orifice (the vein) or within the central volume (the tissue level) (Axel, 1980). This assumption justifies the use of the first moment of C(t) measured in the tissue in most of the MR perfusion studies, but the accuracy of this approach has already been questioned (Hamberg et al., 1993; Weisskoff et al., 1993).

The definition of MTT involves the transit of an idealized narrow square-wave bolus from an instantaneous delta function injection. Practically, however, the intravenous injection of the MR contrast agent has finite duration and furthermore will be delayed and prolonged before reaching the artery supplying the observed tissue. Consequently, the actual observed tissue function $C_{obs}(t)$ is the convolution of the input function $C_a(t)$ and the residue function $C_{tissue}(t)$. the tissue concentration-time curve that would have been achieved with an ideal instantaneous injection.

$$C_{obs}(t) = C_a(t) * C_{tissue}(t)$$

To find $C_{tissue}(t)$, $C_{obs}(t)$ should be deconvolved with $C_a(t)$. However, the first moments of convolved functions are generally additive (Zierler, 1962), so that

$$MTT_{obs} = MTT_a + MTT_{tissue}$$

which gives a simpler method for correcting MTT, as long as $C_a(t)$ and its associated MTT_a can be measured. This can be done from the MR signal of an artery feeding the organ and appearing in the scan plane.

Using an intravascular contrast agent, MTT_{tissue} only defines the flow per unit vascular volume. To calculate the flow per unit tissue volume, the fraction of vascular volume in the tissue (CBV for cerebral perfusion studies) must be determined. The absolute value of CBV can be calculated by integrating the observed tissue concentration-time curve and normalizing to the integrated arterial input function, or to the integrated draining vein function $C_v(t)$, as it can be shown that $\int C_a(t)dt = \int C_v(t)dt$ (Axel, 1980). Thus

$$CBV = \frac{\int_0^\infty C_{obs}(t)dt}{\int_0^\infty C_{alv}(t)dt} \quad (3)$$

$C_v(t)$ can be measured in a venous structure appearing in the scan plane, such as the superior sagittal sinus. Without knowing the area under $C_a(t)$ or $C_v(t)$, a constant value may be assumed and relative values of CBV may be estimated based on the measurement of $\int C_{obs}(t)dt$ alone. For comparisons of CBV measured under different physiological situations. the assumption of a constant input function may not always hold. Similarly, the knowledge of $C_a(t)$ is required for the calculation of an absolute value of the tissue flow, as MTT_{obs} (in the venous output or in the tissue itself as discussed above) must be corrected by MTT_a. Other models incorporating

the input function have been proposed (Larson, et al., 1994).

MATERIALS AND METHODS

Animal model

All animal procedures were approved by the University of Texas Medical Branch Animal Care and Use Committee. Male Sprague-Dawley rats (310 to 490 g body weight) were initially anesthetized with 4% halothane in balanced breathing air (30% O_2/70% N_2). Rats were then intubated and mechanically ventilated using a modified compressed air-powered clinical pressure ventilator (Monaghan, Littleton, CO, U.S.A.), with halothane maintained at 0.6 to 1% during the surgery and MRI procedures. A femoral arterial cannula was inserted for blood gas determination and MABP monitoring. A cannula was inserted in the femoral vein to deliver the bolus of gadopentetate dimeglumine (Magnevist, Berlex Imaging, Wayne, NJ. U.S.A.), the intravascular MR contrast agent, and of N^G-Nitro-L-arginine methyl ester (L-NAME). A second intravenous femoral cannula was inserted when necessary for SNP (Elkins-Sinn, Cherry Hill, NJ. U.S.A.) or phenylephrine (Elkins-Sinn) continuous infusion. The rectal temperature was kept at 37°C with a warm-water blanket during surgery. During MRI, the rat temperature was maintained by circulating warm air through the magnet bore.

Total preparation time was about 2 hours. Then two perfusion measurements by bolus-tracking T2*-weighted sequences were performed at 30-minute intervals under control conditions. Considering the stability and reproducibility of the dynamic susceptibility contrast MRI method for sequential perfusion measurements, previous investigations showed a persistent modification of the signal intensity after a second injection of gadoteridol (ProHance, Bracco Diagnostics, Princeton, NJ, U.S.A.) (Levin et al., 1995). Consequently, only the second perfusion measurement performed under control conditions was used for comparison with the measurements performed after drug administration.

In a first group of rats (L-NAME, n = 9), a first intravenous bolus injection of 3 mg/kg L-NAME was given, followed after 1 hour by a second intravenous injection of 15 mg/kg L-NAME. Perfusion measurements were performed 30 and 60 minutes after each L-NAME injection. For L-NAME + SNP experiments, two rats underwent a perfusion measurement 30 minutes after a 20 mg/kg L-NAME injection. This dose was selected because reversal of CBF effect of 20 mg/kg L-NAME by SNP has been reported (Smith et al., 1995). Then a continuous infusion of SNP (0.1 mg/mL, 20 to 40 μL/kg/min) was started at the lower dose and gradually increased until MABP was restored to its pre- L-NAME value. The infusion was then continued at this rate. A perfusion measurement was performed after a stable MAP was achieved. For the phenylephrine experiments (n = 2), phenylephrine infusion (0.25 mg/mL, 5 to 50 μL/kg/min) was started after the control perfusion measurements. Once a new steady state at high MABP was achieved, a perfusion measurement was performed. Then the phenylephrine infusion was discontinued resulting in a decrease in MABP. We waited 30 minutes between measurements.

At the end of the experimental protocol, the rats were killed with an intravenous injection of 60 mg pentobarbital sodium (Nembutal, Abbott Laboratories, North Chicago, IL, U.S.A.).

Magnetic resonance imaging experiments

The proton MRI experiments were performed using a 4.7 Tesla, 33-cm horizontal bore magnet (SISCO/Varian, Palo Alto, U.S.A.). Excitation and signal detection were achieved with a 6-cm surface coil. Twelve contiguous spin-echo T2-weighted coronal images were acquired, with a 2-second repetition time, 65-millisecond echotime, 128 phase-encoding steps, 4-cm field of view, and 1.7-mm slice thickness. In this set of images, the coronal plane corresponding approximately to stereotaxic coordinates +7.6 mm interaural and −1.4 mm bregma, Paxinos coordinates (Paxinos and Watson, 1986) was chosen for the single-slice bolus tracking experiments. A T2*-sensitive fast low-angle shot pulse sequence (Frahm et al., 1987) was used, with 11- millisecond repetition time, 2.5 millisecond echotime, 8-cm field of view, and 1.9-mm slice thickness. Thirty T2*-weighted images were recorded consecutively. For each perfusion measurement, a first reference movie of 30 T2*-weighted images with a matrix 128 × 128 and 2 averaged acquisitions was performed. Then a second movie was acquired with 30 T2*-weighted images with a matrix of 128 × 64 and 1 acquisition, resulting in a recording time of 0.7 seconds per image. During this second acquisition, a bolus of 0.3 mmol/kg of gadopentetate dimeglumine was injected into the femoral vein synchronously with the start of the 6th image, over a 1.5 second interval. A reconstruction algorithm used the higher phase-encoding steps of the high spatial resolution reference movie and the lower phase-encoding steps of the low spatial but high temporal resolution bolus-tracking movie to yield a 128 × 128 matrix image where the signal intensities corresponded to the bolus-tracking movie.

Data analysis

The time-course of the signal intensity was calculated for each pixel. Signal intensities were transformed into relative concentrations C(t) of the tracer (Belliveau et al., 1990).

$$C(t) = \frac{-1}{TE\, k_1} \ln \frac{S(t)}{S_0} \qquad (4)$$

where TE is the echotime, S_0 is the baseline signal intensity, S(t) is the time-dependent signal intensity, and k_1 is a proportionality factor related to tissue, pulse sequence, and field strength characteristics (Quast et al, 1993).

$C_b(t)$ was determined for a region of interest covering the whole brain tissue, excluding the sagittal sinus and the basal area displaying the large vessels. To measure $C_a(t)$, a single pixel was chosen in an area corresponding to a transversally cut artery in the circle of Willis. Compared to the brain tissue, the arteries were characterized in T2*-weighted images by a higher baseline signal intensity, and an earlier decrease and recovery of the signal intensity with the passage of the contrast agent. To minimize the partial volume effect, the arterial pixel with the highest intensity achieved was chosen. $C_v(t)$ was measured in the superior sagittal sinus both in a single pixel chosen for its highest value of $\int C_v(t)dt$, and in a region of interest covering the whole area of the superior sagittal sinus.

Concentration-time curves from dynamic-susceptibility contrast MR images are usually fitted to a gamma variate model. In the present study, the data did not conform readily to this model, especially after L-NAME administration, which deformed the curve, and, in the sagittal vein, showed multiple compartments. For this reason, numerical integration was used. $C_b(t)$, $C_a(t)$, and $C_v(t)$ were integrated between the time of arrival (TOA) of the contrast agent, defined as the initial point of significant deflection from baseline, and the end of the first pass of the contrast agent, the signal corresponding to recirculation being cut off. This calculation yielded the values of $\int C_b(t)dt$, $\int C_a(t)dt$, and $\int C_v(t)dt$. For the calculation of MTT by Equation 2, t = 0 was set at the time of injection of the contrast agent. To better characterize the shape of the concentration-

time curves, the MTT corrected for TOA was also calculated, and called MTTsh. For the venous curve, MTT_v, TOA_v, and $MTTsh_v$ were calculated with the single pixel measurement of $C_v(t)$, which minimized the partial volume effect, but $\int C_v(t)dt$ was calculated from a combined group of several pixels within the superior sagittal sinus, as this value of the integral was more reproducible than in a single pixel.

Although the purpose of this experiment was to assess modifications of the input function, calculations of CBV and CBF were attempted. Cerebral blood volume was calculated according to Equation 4, by the ratio of $\int C_b(t)dt$ to $\int C_v(t)dt$ (preferred to $\int C_a(t)dt$ whose variability was higher because of the single pixel measurement of $C_a(t)$). The results (mean and SD) were converted to percents of the mean value at baseline. MTT_{tissue} was corrected for the input function according to Equation 3, by subtracting MTT_a from MTT_v, the MTT of the outflow concentration-time curve. Cerebral blood flow (CBF) was calculated by the ratio of CBV to MTT_{tissue}. Because of limited temporal resolution, the variations of MTT_{tissue} were large compared with the small absolute values of this parameter used as a denominator; therefore, the actual calculation of individual CBF values was subjected to large errors. However, CBF could be evaluated as the ratio of the mean values of CBV and MTT_{tissue}. The SD of those ratios were calculated according to Raj (1968). The absolute values of the ratios and SD were converted to percents of the ratio calculated at baseline.

Data are expressed as mean ± SD. Statistical differences were obtained using a repeated measurement analysis of variance, followed by a Bonferroni t-test for multiple group comparisons, using the statistical software SigmaStat 1.0 (Jandel, San Rafael, CA, U.S.A.). A value of $P < 0.05$ was considered to be significant for drug effects.

RESULTS

Blood gas parameters did not change immediately before and after MR imaging experiments in the L-NAME (n = 9), L-NAME + SNP (n = 2), and phenylephrine (n = 2) groups. Those parameters remained in the physiological range for rats during the whole observation period (Baker et al., 1979). L-NAME injection induced a significant increase of MABP (Table 1). The MABP for the SNP and phenylephrine-treated rats are also shown.

Representative images out of a sequence of 30 dynamic susceptibility contrast images acquired during baseline conditions are displayed in Fig. 1 for illustration of the time-course of signal intensity in arteries, brain tissue, and sagittal sinus. Signal intensities were converted to concentration-time curves (C(t)) of the contrast agent.

The effect of 3 mg/kg and 15 mg/kg L-NAME on perfusion parameters are presented in Fig. 2. After injection of L-NAME, MTT_a increased significantly, reflecting an augmentation of both TOA_a (significant 30 minutes after the low dose, and 30 and 60 minutes after the high dose of L-NAME), and $MTTsh_a$ (significant only after the high dose). $\int C_a(t)dt$ did not change significantly, although a trend toward an increase was suggested. The arterial curve seemed especially affected by limited spatial resolution. An increase of MTT, TOA, and MTTsh was observed after L-NAME in the venous and the brain functions, reaching statistical significance after 15 mg/kg L-NAME, except that TOA_v already significantly increased 30 minutes after the low dose, and $MTTsh_v$ did not reach statistical significance. Unlike $\int C_a(t)dt$, $\int C_v(t)dt$ increased significantly after 15 mg/kg L-NAME, but the increase of $\int C_b(t)dt$ was not significant.

In the two animals tested, SNP infusion reversed the effects of 20 mg/kg L-NAME on MTT, TOA, and MTTsh of the arterial, brain, and vein functions (Fig. 3). Sodium nitroprusside also reversed the increase of $\int C_v(t)dt$ induced by L-NAME. In the two animals studied, MTT, TOA, and MTTsh did not increase, or even decreased during phenylephrine infusion in the arterial, brain, and vein functions (Fig. 4). Compared to L-NAME, phenylephrine had also an opposite action on $\int C_v(t)dt$, which decreased. Stopping phenylephrine infusion reversed these parameters.

The purpose of this study was to illustrate that the input function can be affected by changes in systemic physiological conditions. However, we did preliminary calculations of CBV and CBF with and without consideration of the input function and evaluated the direction of effects. Cerebral blood volume values were calculated by Equation 4. The results are presented as percent of the baseline value. Cerebral blood volume tended to de-

TABLE 1. *Mean arterial blood pressure*

L-NAME (n = 9)		L-NAME + SNP (n = 2)			Phe (n = 2)		
	Mean ± SD		Rat 1	Rat 2		Rat 1	Rat 2
MABP (mm Hg)							
Baseline	89 ± 7	Baseline	79	85	Baseline	89	98
30 min after 3 mg/kg L-NAME	117 ± 7*	30 min after 20 mg/kg L-NAME	118	128	Phe	130	142
1 h after 3 mg/kg L-NAME	115 ± 12*	L-NAME + SNP	84	105	Discontinuation of Phe	82	100
30 min after 15 mg/kg L-NAME	124 ± 9*						
1 h after 15 mg/kg L-NAME	122 ± 11*						

L-NAME, N-nitro-L-arginine methyl ester; Phe, phenylephrine; SNP, sodium nitroprusside.
* Significantly higher than baseline value (Bonferroni t-test for multiple comparisons, $P < 0.05$)

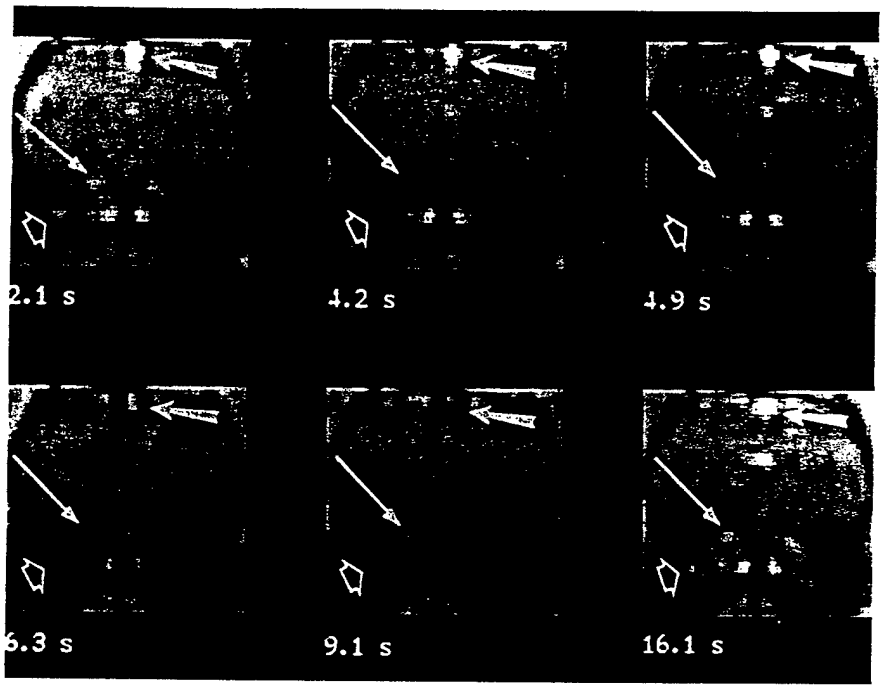

FIG. 1. Dynamic susceptibility contrast images chosen out of 30 gradient-echo images acquired every 0.7 seconds before and after injection of 0.3 mmol/kg gadopentetate dimeglumine. The first image corresponds to 2.1 seconds after injection of the contrast agent, which did not reach the brain yet. The arteries (intracerebral artery: long thin arrow, and artery of the Willis circle: short empty arrow), and the sagittal sinus (thick arrow) are brighter than the cerebral tissue. The arteries become darker 4.2 seconds after injection, followed by the cerebral parenchyma at 4.9 seconds. After 6.3 seconds, the contrast agent reached the sagittal sinus. At 9.1 seconds, the first pass of the contrast agent had left the arteries, which reappeared brighter, while the sagittal sinus was still dark. After 16.1 seconds, the contrast agent had also left the sagittal sinus.

crease after L-NAME injection (95 ± 38% and 86 ± 29% 30 minutes after 3 mg/kg and 15 mg/kg L-NAME, respectively), but did not reach statistical significance because of variance. Sodium nitroprusside infusion restored CBV after the decrease induced by 20 mg/kg L-NAME or increased it to higher values than during the control period (from 81 and 83% to 95 or 137%, respectively in 2 rats). Phenylephrine infusion increased CBV which came back to baseline when the phenylephrine infusion was discontinued (170 and 115% to 105 and 95%, respectively in 2 rats). If the input function correction of $\int C_v(t)dt$ had not been taken into account, the values of $\int C_b(t)dt$ would have shown a trend for increased CBV after 15 mg/kg L-NAME (114 ± 20% after 30 minutes and 108 ± 21% after 60 minutes), contrary to the results found by Equation 4, where CBV is corrected for $\int C_v(t)dt$ (86 ± 29% after 30 minutes and 90 ± 38% after 60 minutes).

MTT_{tissue} was calculated by subtracting MTT_a from MTT_v (Equation 3). There was large interindividual variations when MTT_{tissue} were calculated, but interesting trends were observed. MTT_{tissue} decreased slightly after 3 mg/kg L-NAME (from 1.59 ± 0.61 seconds at baseline to 1.47 ± 0.89 and 1.27 ± 1.02 seconds at 30 and 60 minutes after 3 mg/kg L-NAME respectively) but increased to 2.07 ± 1.06 and 2.00 ± 1.46 seconds at 30 and 60 minutes after 15 mg/kg L-NAME, respectively. Considering that CBF is the ratio of CBV to MTT_{tissue} (Equation 1), the decreased CBV and increased MTT_{tissue} observed after 15 mg/kg L-NAME suggests that CBF was decreased in these conditions. Figure 5 illustrates that the decrease of CBF after 15 mg/kg L-NAME would not have been appropriately recorded if the input function had not been taken into account: CBV/MTT_{tissue} decreased to 66 ± 17% and 72 ± 25% at 30 and 60 minutes after 15 mg/kg L-NAME compared with 100 ± 22% at baseline, while $\int C_b(t)dt/MTT_b$ remained at 93 ± 9% and 91 ± 8% for the same conditions compared with 100 ± 5% at baseline. The smaller numbers of data in L-NAME + SNP and phenylephrine groups did not allow the calculation of mean MTT_{tissue} and CBF, but the individual values of CBF were calculated. They indicated that SNP reversed the decrease of CBF induced by the L-NAME injection, and that phenylephrine infusion increased CBF, which decreased again when the phenylephrine infusion was stopped (Table 2).

DISCUSSION

The present study showed that changes in the physiological state of the rats investigated here can modify the input function. The assumption of a constant input function allows the comparison of different brain regions within one individual at a time. Under constant physi-

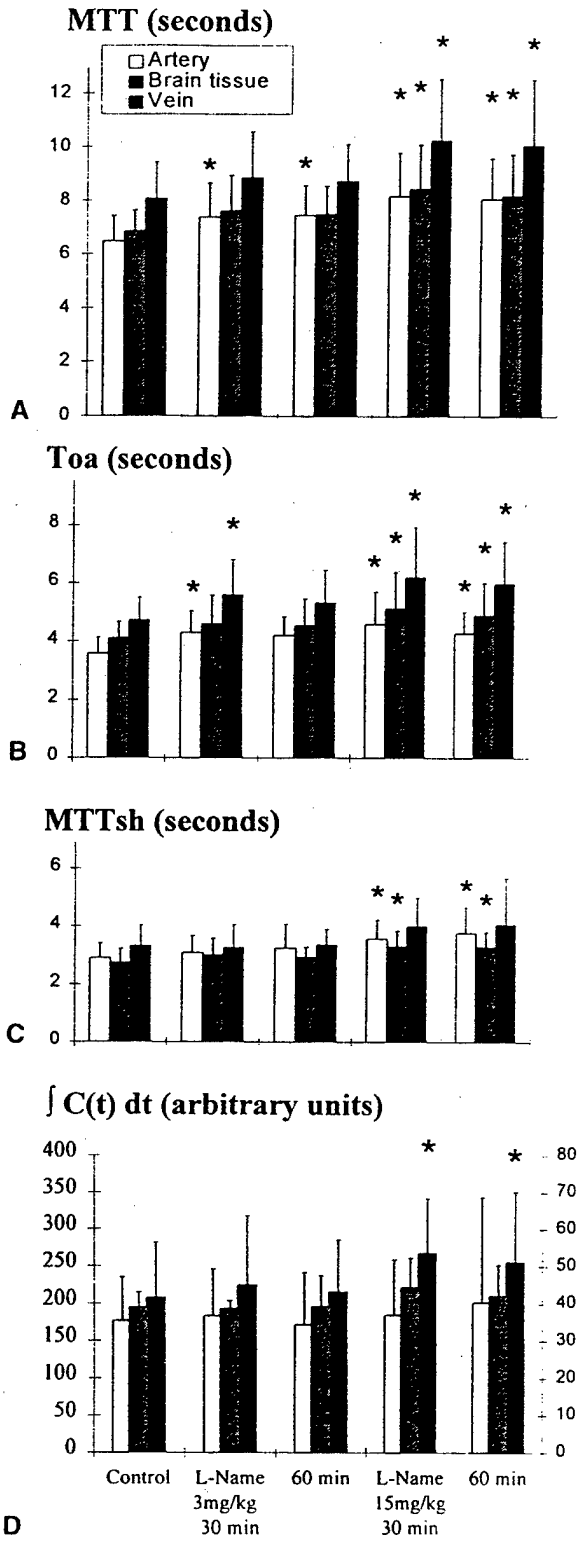

FIG. 2. **(A)** Mean transit time (MTT), **(B)** time of arrival (Toa), **(C)** MTT corrected for Toa (MTTsh), and **(D)** area of the arterial, brain tissue, and venous concentration-time curves ($\int C_v(t)dt$) in control conditions and after N_ω-Nitro-L-arginine methyl ester (L-NAME) injections. *Parameters significantly increased compared with the control values (Bonferroni t-test for multiple comparisons, $P < 0.05$). In D, the area under the brain curve is indicated on the right side axis.

ological conditions, the validity of this assumption has already been shown by others (Wittlich et al., 1995). This previous investigation also proved that input function can be used as a constant for a group of genetically homogeneous animals, provided the physiological and the imaging conditions of the animals remain unchanged. In an MR human study, input function measured in arteries visible on the scan plane showed a strong interindividual variation, although none of the investigated subjects had a history of intracranial abnormality (Rempp et al., 1994), suggesting that among humans input function differs even if the individuals are subjected to the same conditions.

The modifications of the concentration-time curves observed in the brain tissue and in the sagittal vein after administration of L-NAME were partly caused by their convolution with a modified input function. This was illustrated by the decreased calculated CBV, resulting from an increase of the area under $C_b(t)$, but a stronger increase of the area under $C_v(t)$, used to correct $\int C_b(t)dt$. Ignoring the correction by the input function would have led to the calculation of increased CBV after 15 mg/kg L-NAME.

With this correction, the CBF calculated for 30 minutes after 15 mg/kg L-NAME was reduced to 66 ± 17% of the baseline value. This result is consistent with previous laser Doppler measurements showing that CBF decreased to 67.6 ± 8.1% in rat cerebral cortex after 15 mg/kg L-NAME (Prado et al., 1992) or to 74 ± 2% after 20 mg/kg L-NAME (Smith et al., 1995), with radioactive microsphere measurements showing a global CBF decreased to 74 ± 12% after 20 mg/kg L-NAME (Huang et al., 1994), and with [^{14}C]-iodoantipyrine autoradiography showing a decrease of CBF between 53 ± 3% and 86 ± 6%, depending on the cortical area, after 30 mg/kg L-NAME (Kelly et al., 1994).

The shape and TOA of the input function depends on the way the intravenous contrast agent bolus has been delayed and deformed between the site of injection and the observed feeding artery. The lower dose of 3 mg/kg L-NAME induced a 31% increase of the MABP, close to the 39% increase caused by the higher dose, but less consistent modifications of the concentration-time curves, suggesting that those modifications were more related to the level of nitric oxide synthase inhibition rather than to the consequent hypertension. The reversal of these modifications by SNP infusion would thus be more likely caused by the direct supply of nitric oxide synthase rather than to the reversal of MABP changes. Phenylephrine infusion caused a comparable hypertension, but did not increase TOA, MTT or MTTsh, or even decreased those parameters.

Modifications of cerebral vascular resistance (CVR), and the relative value of CVR compared with the vascular resistance in other organs, could affect the bolus dis-

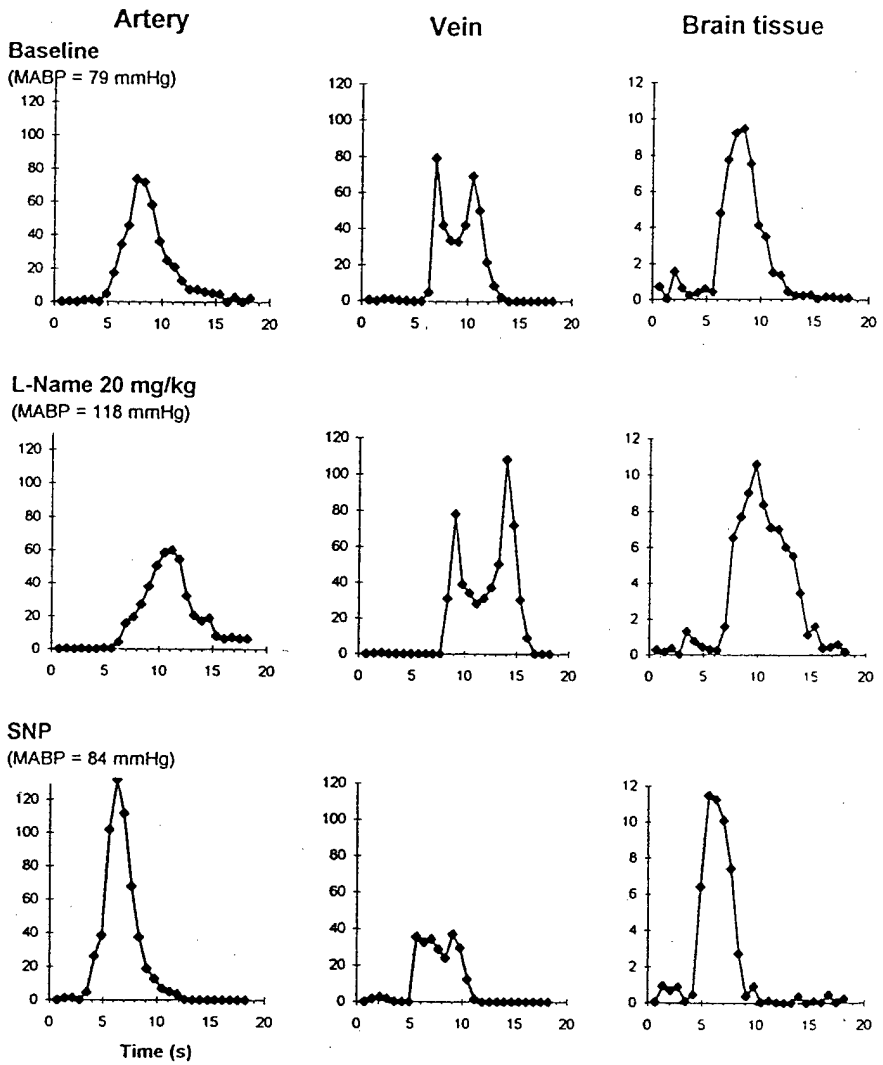

FIG. 3. Arterial, brain tissue, and venous concentration-time curves of a rat administered 20 mg/kg L-NAME followed by sodium nitroprusside (SNP) infusion (0.1 mg/mL, 20 to 40 μL/kg/min). L-NAME increased the MABP, the time of arrival, and mean transit time of all curves, and SNP reversed these modifications. These results indicate that the alterations seen with L-NAME (Fig. 2) were not simply caused by the effect of serial injections.

tribution to brain during its course in the aorta and likely influence the size and shape of the cerebral input function. Calculating CVR as the ratio of MABP to CBF, we found that CVR reached 211% of the control value 30 minutes after 15 mg/kg L-NAME. This increase of CVR induced by L-NAME injection is comparable with the values calculated with MABP and CBF values of previous studies: CVR = 183% (Huang et al., 1994) or 164% (Smith et al., 1995) after 20 mg/kg intravenously, and 156 to 253% depending on the cortical area after 30 mg/kg intravenously (Kelly et al., 1994). However, during phenylephrine infusion, our calculations suggested that CBF actually increased, as would be the case if there was a breakthrough of autoregulation. Consequently, the hypertension induced by phenylephrine infusion was accompanied by a smaller CVR increase than the hypertension induced by L-NAME. The distribution of the contrast agent bolus to the brain would thus be less (or differently) affected, contributing to the difference in input function modifications between both conditions. The trend of increasing CBF with phenylephrine, suggesting a partial breakthrough of autoregulation, would be in agreement with previous laser Doppler measurements of increased CBF in urethane anesthetized rats subjected to phenylephrine (Tsai et al., 1989), and with the previously reported impairment of the cerebral autoregulation during phenylephrine infusion in halothane-anesthetized rabbits (Mutch et al., 1990). The dependence of input function on CVR relative to other vascular beds would also suggest that the measurement of input function in blood samples withdrawn from a peripheral artery, as practiced in single pass CBF measurements techniques, could be misleading if it does not reflect the input function in the carotid bed.

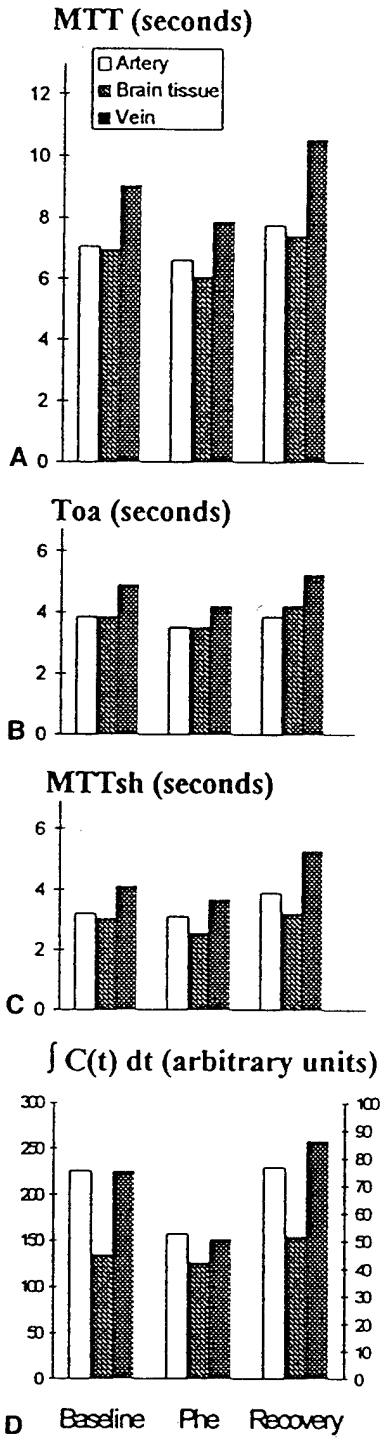

FIG. 4. Mean values of **(A)** mean transit time (MTT), **(B)** time of arrival (Toa), **(C)** MTT corrected for Toa (MTTsh), and **(D)** area of the arterial, brain tissue, and venous concentration-time curves ($\int C_v(t)dt$), during control conditions (baseline), during phenylephrine infusion (Phe), and after stopping phenylephrine (recovery), for the two animals studied. In D, the area under the brain curve is indicated on the right side axis.

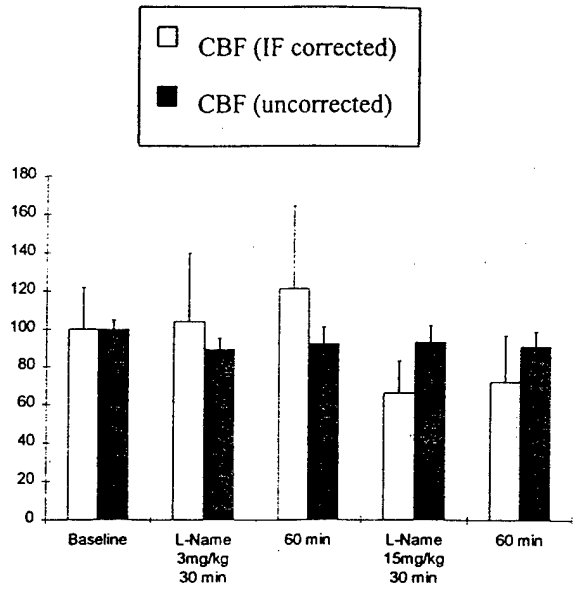

FIG. 5. Assessment of CBF changes after L-NAME injections, with correction by the input function (CBV/MTT_{tissue}) or without this correction ($\int C_v(t)dt$). The displayed results are the ratios of mean values of CBV and MTT_{tissue}, $\int C_v(t)dt$ and MTT_b. Error bars are the SD of these ratios (Raj, 1968).

The indicator dilution theory states that the integrated input function = $\int C_a(t)dt = \int C_v(t)dt$, but the present study found larger areas under the vein curves than under the arterial curves. This finding may be explained by a partial volume effect affecting the arterial pixels more than the venous pixels, because of the smaller size of the arteries compared with the superior sagittal sinus. Although showing larger areas under the curves, $\int C_v(t)dt$ was still apparently underestimated. The ratio of $\int C_b(t)dt$ to $\int C_v(t)dt$ yielded an absolute value of 21 ± 2% for CBV under control conditions, which is too high compared with the values measured previously in rat brains by other methods (3.4% (Shockley, 1988; Kent, 1989)). Thus, volume averaging may not be the only cause of signal loss and both flow and susceptibility effects may be occurring.

Most previous CBF studies calculated CBF by the ratio of CBV to the MTT of the tissue concentration-time curve by first pass bolus tracking. But Hamberg et al. (1993) and Weisskoff et al. (1993) expressed reservations about the use of MTT measured with MRI tissue concentration-time curves for the calculation of CBF by the central volume principle. Weisskoff et al. showed that the first moment of the concentration-time curve of the tracer at the outlet of the system, which relates CBV to CBF, may not be substituted by the first moment of the concentration-time curve measured in the tissue, R(t), which expresses the amount of the bolus that remains in the tissue. They showed that the first moment of R(t) depends on the topology of the vessels. In the present study, we calculated CBF by using MTT_v, the first mo-

TABLE 2. *Estimation of cerebral blood flow**

L-NAME (n = 9)		L-NAME + SNP (n = 2)			Phe (n = 2)		
	Mean CBV / Mean MTT_{tissue} ± SD†		Rat 1	Rat 2		Rat 1	Rat 2
Baseline	100 ± 22	Baseline	100	100	Baseline	100	100
30 min after 3 mg/kg L-NAME	104 ± 36	30 min after 20 mg/kg L-NAME	64	39	Phe	202	269
1 h after 3 mg/kg L-NAME	121 ± 43	L-NAME + SNP	71	74	Discontinuation of Phe	56	105
30 min after 15 mg/kg L-NAME	66 ± 17						
1 h after 15 mg/kg L-NAME	72 ± 25						

L-NAME, N_ω-nitro-L-arginine methyl ester; Phe, phenylephrine; SNP, sodium nitroprusside; CBV, cerebral blood volume; MTT, mean transit time.
* Ratios and SD values were converted in percents of the estimated baseline CBF.
†SD on the ratio of mean CBV to mean MTT_{tissue} were calculated according to Raj (1968). Statistical differences between the estimated CBF were not determined because those values were not the means of individual measurements.

ment of the concentration-time curve in the superior sagittal sinus, i.e. the MTT actually measured at the outlet of the system, in agreement with the central volume principle. But the drawback of using MTT_v (corrected by MTT_a to take into account the deconvolution with input function), is that it gave only the value of a global CBF, and did not allow the calculation of regional CBF in the different anatomical structures of the brain.

Weisskoff et al. (1993) also suggested that the ratio of MTT measured by R(t) could give a reasonable estimate of the relative flow changes either between two regions of the brain with similar vascular physiology, or between the same region before and after some perturbation, if the perturbation changes the vascular structure only moderately. It must be kept in mind also, that in this case, MTT from the input bolus must be subtracted from MTT of R(t). In our study, this estimation could not be calculated because of too small differences between MTT_b and MTT_a. In addition to relative poor temporal resolution, this effect was probably caused by underestimation of MTT_b, because of the low signal to noise ratio of $C_b(t)$ which could have hidden the extremes of the curve into an apparent baseline.

In conclusion, significant changes of the brain input function were found after intravenous injection of L-NAME. These input function changes were not simply caused by an effect on MABP, but possibly involved the complex effects of L-NAME on vascular resistance in the brain and other organs. The important implications of input function modifications for accurate measurements of CBV and CBF were illustrated by calculating their changes after L-NAME injection.

Acknowledgments: We thank Douglas Barstow, Dr. Pitchaiah Mandava, Bernard Kim, Charmaine Rea, and Benoit Leroy for their technical help; and Tatsuo Uchida, MA, Office of Biostatistics, University of Texas Medical Branch, for important statistical advice.

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JOURNAL OF NEUROTRAUMA
Volume 14, Number 4, 1997
Mary Ann Liebert, Inc.

L-Arginine and Superoxide Dismutase Prevent or Reverse Cerebral Hypoperfusion after Fluid-Percussion Traumatic Brain Injury

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ABSTRACT

To determine whether treatment with L-arginine or superoxide dismutase (SOD) would prove effective in reducing cerebral hypoperfusion after traumatic brain injury (TBI), we measured cerebral blood flow (CBF) using laser Doppler flowmetry (LDF) in rats treated before or after moderate (2.2 atm) fluid-percussion (FP) TBI. Rats were anesthetized with isoflurane and prepared for midline FP TBI and then for LDF by thinning the calvaria using an air-cooled drill. Rats were then randomly assigned to receive sham injury, sham injury plus L-arginine (100 mg/kg, 5 min after sham TBI), TBI plus 0.9% NaCl, TBI plus L-arginine (100 mg/kg, 5 min post-TBI), TBI plus SOD (24,000 U/kg pre-TBI + 1600 units/kg/min for 15 min after TBI), or TBI plus SOD and L-arginine. A second group of rats received TBI plus saline, L-, or D-arginine (100 mg/kg, 5 min after-TBI). After treatment and TBI or sham injury, CBF was measured continuously using LDF for 2 h and CBF was expressed as a percent of the preinjury baseline for 2 h after TBI. Rats treated with saline or D-arginine exhibited significant reductions in CBF that persisted throughout the monitoring period. Rats treated with L-arginine alone or in combination with SOD exhibited no decreases in CBF after TBI. CBF in the SOD-treated group decreased significantly within 15 min after TBI but returned to baseline levels by 45 min after TBI. These studies indicate that L-arginine but not D-arginine administered after TBI prevents posttraumatic hypoperfusion and that pretreatment with SOD will restore CBF after a brief period of hypoperfusion.

Key words: cerebral circulation; free radicals; head trauma; ischemia; L-arginine; laser Doppler; nitric oxide

INTRODUCTION

TRAUMATIC BRAIN INJURY (TBI) in humans results in reduced cerebral blood flow (CBF) in the first few hours after injury (Bouma et al., 1991, 1992). Although the role played by posttraumatic hypoperfusion in this pathophysiology is not known, evidence of ischemia in most TBI patients (Graham et al., 1978) suggests that reductions in CBF may be important contributors. Early posttraumatic hypoperfusion occurs after experimental TBI in rats (Yuan et al., 1988; Yamakami and McIntosh, 1989, 1991). Moderate central or midline fluid-percussion

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(FP) TBI in rats decreased CBF in the cerebral hemispheres, brain stem, and cerebellum (Yuan et al., 1988). Moderate lateral TBI produced similar decreases in CBF in the brain stem, cerebellum, diencephalon, and frontal and parietal cortices on both (i.e., injured and uninjured) sides of the rodent brain (Yamakami and McIntosh, 1989, 1991). Most recent measurements of CBF after FP using laser Doppler flowmetry (LDF) confirm earlier microsphere CBF measurements (Muir et al., 1992, 1995).

The causes of significant reductions in CBF after TBI in either patients or experimental animals are not known. Posttraumatic hypoperfusion may result from impairment or destruction of a cerebral vasodilatory mechanism or mechanisms. The endothelium-dependent relaxing factor nitric oxide (NO) is one such cerebral vasodilator (Furchgott and Zawadzki, 1980; Ignarro et al., 1987). Evidence that inhibition of NO synthesis decreases CBF (Beckman et al., 1991; Tamaka et al., 1991; DeWitt et al., 1992; Pelligrino et al., 1993) suggests a resting cerebral vasodilator "tone" owing to the continuous production of NO. Nitric oxide is inactivated by contact with free radicals such as superoxide (Rubanyi and Vanhoutte, 1986). Superoxide anion radicals are produced by FP TBI (Wei et al., 1981; Fabian et al., 1995), perhaps as a byproduct of trauma-induced increases in prostaglandin synthesis (DeWitt et al., 1988). The superoxide radical may inactivate NO, resulting a reduced CBF after TBI. Free radicals contribute to the pathophysiology of TBI, as the cyclooxygenase inhibitor indomethacin and the free radical scavenger superoxide dismutase (SOD) reduce cerebral vascular dysfunction and endothelial damage after TBI (Wei et al., 1981). Treatment with L-arginine, the substrate for the enzyme NO synthase, increases CBF and reduces infarct volume due to focal ischemia (Morikawa et al., 1992a,b), suggesting that increased production of NO might increase CBF and reduce ischemic damage. In contrast, L-arginine increased infarct size in a suture model of middle cerebral artery occlusion in a rat (n = 3) and L-arginine did not reduce infarct volume or increase CBF after a photothrombotic ischemic insult (Prado et al., 1996). Therefore, the effects of L-arginine on CBF after cerebral ischemia are uncertain and L-arginine effects on CBF after TBI are unknown. To determine whether L-arginine would improve CBF after rodent TBI, we measured CBF using LDF in rats treated with L-arginine or SOD or the combination after FP TBI.

MATERIALS AND METHODS

Animal Preparation

All experimental protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas Medical Branch. Male Sprague-Dawley rats weighing 350–400 g were anesthetized with isoflurane in an anesthetic chamber, intubated, and mechanically ventilated with 1.5–2.0% isoflurane in O_2: room air (70:30) using a volume ventilator (EDCO Scientific, Chapel Hill, NC). Polyethylene cannulae were placed in a femoral artery and vein for drug infusion and arterial pressure monitoring, respectively. Rectal temperature was monitored using a telethermometer (Yellow Springs Instruments, Yellow Springs, OH) and maintained using a thermostatically controlled water blanket (Gaymar, Orchard Park, NY). Rats were prepared for midline FP TBI was previously described (Dixon et al., 1987). Briefly, the rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4-mm hole was trephined into the skull over the sagittal suture approximately midway between lambda and bregma, and a modified Luer-Lok syringe hub was placed over the exposed dura and bonded in place with cyanoacrylic adhesive and covered with dental acrylic. Rats were then prepared for placement of the laser Doppler flow probe (see below). Isoflurane was lowered to 1.5%, and the rats were connected to the trauma device, and subjected to moderate (2.2 atm) TBI.

Laser Doppler Flowmetry

CBF was measured using LDF. The anesthetized rats were surgically prepared for measurement of relative perfusion as described elsewhere (Haberl et al., 1989). Briefly, the left calvaria lateral and slightly posterior to the injury adapter (see above) was thinned using an air-cooled drill (Dremel, Racine, WI). Using an electrode holder on a stereotaxic headholder (Stoelting Co., Wood Dale, IL), a fiberoptic needle probe (Perimed, Stockholm, Sweden) was placed over the thinned parietal calvaria and carefully positioned away from large vessels visible in the remaining calvaria. The probe emits monochromatic red light (632.8 nm), which is reflected by moving erythrocytes. The power and frequency of the reflected signal, monitored by detectors in the needle probe head, are proportional to the blood volume and blood velocity, respectively. Blood velocity is calculated based upon the Doppler shift created by red blood cells moving in the area perfused by the probe laser and reflected back to the receiver in the same probe. Perfusion is calculated as the product of blood volume and velocity in a 1-mm^3 tissue volume under the probe (Haberl et al., 1989). Measurements were recorded on a PeriFlux PF3 Laser Doppler Perfusion Monitor (Perimed). Values for LDF were compared between rats based on a percentage change from baseline values after the experimental procedure.

Experimental Design

Drug treatment. The dose of L-arginine was chosen because, although doses of 300 mg/kg increased CBF and

reduced infarct volume after middle cerebral artery occlusion (Morikawa et al., 1992a; Tofts and Kermode, 1991), L-arginine also increased CBF and pial arteriolar diameter in control rats. A lower dose was used in the present study (100 mg/kg) to minimize the cerebral vasodilatory effects of L-arginine in uninjured rats. The dose of SOD used in the present studies (24,000 U/kg, i.v. bolus, 5 min pre-TBI, plus 1,600 U/kg min for 10 min starting immediately after TBI) has been reported to improve CBF after TBI in rats (Muir et al., 1995).

Experiment 1. Male Sprague-Dawley rats were prepared as described above and randomly assigned to one of the six groups listed below. A sham group (n = 8) was prepared for FP TBI and LDF, connected to the trauma device, and then removed from the device without injury. A saline-treated group (n = 8) was subjected to moderate TBI (2.2 atm) and then infused with 0.9% NaCl (0.1 ml/min for 10 min). The L-arginine group (n = 8) was subjected to moderate TBI and then L-arginine (10 mg/kg/min for 10 min) administration was started 5 min later. An L-arginine sham group (n = 8) received that same dose of L-arginine but no TBI. The SOD-treated group (n = 8) received a 24,000 U/kg bolus 5 min before moderate TBI, followed by 1600 U/kg/min constant infusion for 15 min after TBI (volume = 33 μL/min). A combination group (n = 8) received both SOD (before and after TBI) and L-arginine (after TBI) in the same doses as the individual SOD and L-arginine groups. After treatment and TBI or sham injury, CBF was measured continuously using LDF for 2 h; CBF is expressed as a percent of the preinjury baseline 15, 30, 45, 60, 75, 90, 105, and 120 min after TBI. The experimental design for experiment 1 is summarized in Figure 1.

Experiment 2. Male Sprague-Dawley rats were prepared as described above and randomly assigned to one of three groups. A saline-treated group (n = 5) was subjected to moderate TBI (2.2 atm) and then infused with 0.9% NaCl (0.1 ml/min for 10 min). An L-arginine-treated group (n = 5) was subjected to moderate TBI and then L-arginine (10 mg/kg/min for 10 min) administration was started 5 min later. A third group (n = 5) was treated identically to the L-arginine group except for re-

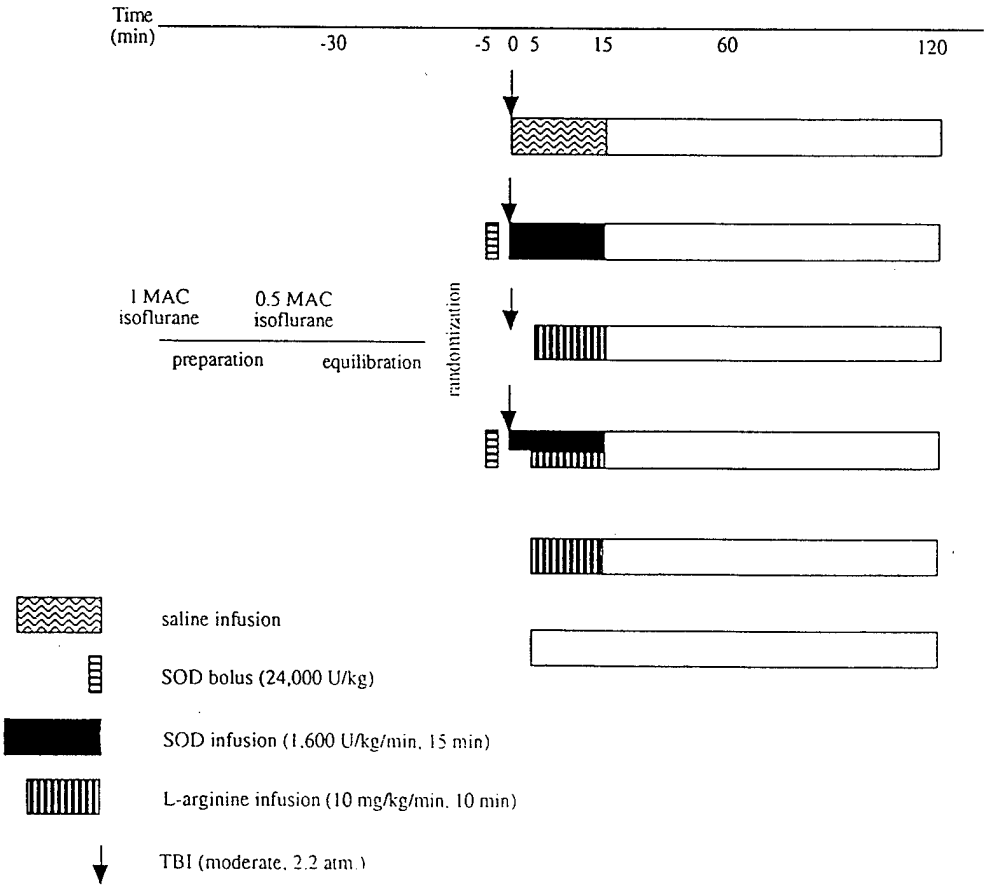

FIG. 1. Schematic of experimental design for experiment 1. The key for the patterned bars is presented in the figure. The open bar represents the monitoring period after TBI and drug infusion. MAC = minimum alveolar anesthetic concentration to produce surgical anesthesia in rats (1.38% isoflurane).

ceiving the inactive stereoisomer, D-arginine (10 mg/kg/min for 10 min), which is not a substrate for NO synthase. After treatment and TBI or sham injury, CBF was measured continuously using LDF for 2 h; CBF is expressed as a percent of the preinjury baseline 15, 30, 60, 90, and 120 min after TBI.

Statistical Evaluations

Data were analyzed using analysis of variance (ANOVA) for a two-factor experiment with repeated measures on time. The two factors were treatment group and time. The number of treatment groups was 6 for experiment 1 and 3 for experiment 2. The time points tested were baseline (before TBI), and 15, 30, 60, 90, and 120 min for CBF and mean arterial pressure (MAP), and before TBI and 60 min after TBI for $PaCO_2$, PaO_2, hemoglobin, and pH. The mean of each time point was compared with the baseline (100% for CBF and the mean for MAP) for each treatment group using Fisher's least significant difference procedure. All values in the text, tables, and figures are expressed as means ± SEM.

RESULTS

Experiment 1

In experiment 1, two rats died during surgical preparation and four rats became progressively hypotensive and died with 15 min of TBI. There were no significant differences in body temperature, $PaCO_2$, PaO_2, or arterial hemoglobin concentrations within any group between pre-TBI baselines and any subsequent measurement interval (Tables 1, 2). In the three groups subjected to moderate TBI, MAP increased within 60 sec of injury and then returned to baseline within 5 min. Except for the acute hypertension period after TBI, there were no significant differences in MAP between the pre-TBI baseline and any measurement interval within any group.

In the sham-injured control rats ($n = 8$) and in the sham-injured rats treated with L-arginine ($n = 8$), CBF remained constant throughout the 2-h measurement interval (Fig. 2). After TBI in the saline-treated group ($n = 8$), CBF decreased significantly ($p < 0.05$) within 15 min after TBI and remained significantly below baseline during the 120-min measurement interval (Figs. 2 and 3). CBF decreased within 15 min after TBI in the SOD-treated group ($n = 8$) but then returned to baseline levels within 60 min after TBI. In the group treated 5 min after TBI with L-arginine ($n = 10$), CBF remained equal to baseline levels throughout the measurement interval. Similarly, CBF did not decrease after TBI in the group treated with the combination of L-arginine plus SOD ($n = 8$).

Experiment 2

In experiment 2, one rat died during surgical preparation and two rats became progressively hypotensive and died within 15 min of TBI. There were no significant differences in body temperature, $PaCO_2$, PaO_2, or arterial hemoglobin concentrations within any group between pre-TBI baselines and any subsequent measurement interval (Table 3). Mean arterial pressure increased within 60 sec of injury and then returned to baseline within 5 min. Except for the brief period of acute hypertension after TBI, there were no significant differences in MAP between the pre-TBI baseline and any measurement interval within any group.

In the saline-treated ($n = 5$) and D-arginine-treated ($n = 5$) groups, CBF decreased significantly ($p < 0.05$) to about 60% of baseline values within 15 min after TBI and remained at these significantly reduced levels for the 120-min monitoring period (Fig. 4). CBF in the group treated with L-arginine ($n = 5$) did not change signifi-

TABLE 1. MEAN ARTERIAL PRESSURE IN SHAM-INJURED RATS TREATED WITH SALINE ($n = 8$) OR L-ARGININE ($n = 8$), OR IN RATS TREATED WITH L-ARGININE ($n = 10$), SOD ($n = 8$) OR L-ARGININE PLUS SOD ($n = 8$) AFTER MODERATE FLUID-PERCUSSION TBI

			Time after TBI					
Variable	*Group*	*Pre-TBI*	*1*	*15*	*30*	*60*	*90*	*120*
MAP[a] (mmHg)	sham	115 ± 5	—	111 ± 5	105 ± 4	106 ± 5	105 ± 4	106 ± 7
	arg-sham	117 ± 6	—	115 ± 7	115 ± 6	115 ± 8	108 ± 8	122 ± 4
	saline	103 ± 7	164 ± 12	110 ± 7	108 ± 8	103 ± 9	97 ± 11	107 ± 11
	SOD	109 ± 7	153 ± 15	116 ± 6	113 ± 5	109 ± 5	109 ± 6	100 ± 8
	L-arginine	115 ± 6	156 ± 13	117 ± 6	117 ± 5	117 ± 5	120 ± 6	106 ± 9
	SODarg	113 ± 2	164 ± 18	118 ± 4	111 ± 4	115 ± 8	113 ± 6	111 ± 8

[a]MAP, mean arterial pressure.

TABLE 2. ARTERIAL BLOOD GASES AND HEMOGLOBIN IN SHAM-INJURED RATS TREATED WITH SALINE (n = 8) OR L-ARGININE (n = 8) OR RATS TREATED WITH L-ARGININE (n = 10), SOD (n = 8), OR L-ARGININE PLUS SOD (n = 8) AFTER MODERATE FLUID-PERCUSSION TBI

Variable	*Group*	*Pre-TBI*	*60 min post-TBI*
$PaCO_2$ (mmHg)	sham	36.6 ± 3.0	33.8 ± 4.7
	arg-sham	31.6 ± 2.0	37.8 ± 3.2
	saline	30.4 ± 4.4	34.3 ± 2.1
	SOD	30.1 ± 4.3	35.8 ± 6.1
	L-arginine	43.2 ± 7.2	41.6 ± 6.9
	SODarg	35.6 ± 3.6	38.2 ± 3.8
PaO_2 (mmHg)	sham	289 ± 9	269 ± 17
	arg-sham	280 ± 11	314 ± 14
	saline	286 ± 13	296 ± 20
	SOD	261 ± 27	287 ± 15
	L-arginine	286 ± 10	278 ± 8
	SODarg	280 ± 19	292 ± 14
pH (mmHg)	sham	7.44 ± 0.03	7.36 ± 0.3*
	arg-sham	7.45 ± 0.02	7.36 ± 0.04*
	saline	7.52 ± 0.01	7.41 ± 0.03*
	SOD	7.44 ± 0.03	7.40 ± 0.04*
	L-arginine	7.44 ± 0.02	7.40 ± 0.01*
	SODarg	7.44 ± 0.02	7.36 ± 0.03*
Hgb (g/dl)	sham	12.5 ± 0.9	12.8 ± 0.6
	arg-sham	13.0 ± 0.6	12.5 ± 0.6
	saline	12.2 ± 0.4	11.7 ± 0.7
	SOD	12.4 ± 0.9	12.4 ± 0.9
	L-arginine	12.8 ± 0.3	11.4 ± 1.4
	SODarg	13.0 ± 0.6	12.7 ± 0.7

*$p < 0.05$ compared with pre-TBI value.

cantly from baseline at any measurement interval after TBI.

DISCUSSION

These studies demonstrated that L-arginine, but not D-arginine, completely prevents posttraumatic hypoperfusion when administered 5 min after moderate FP TBI. L-arginine alone had no effect on CBF. Superoxide dismutase, administered before and after TBI, restored CBF after a 30-min period of hypoperfusion. The combination of L-arginine and SOD prevented posttraumatic hypoperfusion, as did L-arginine alone. Our observations that SOD improves CBF after TBI are consistent with those of Muir et al., who observed that SOD restored CBF nearly to baseline levels within 1 h after moderate FP TBI in rats anesthetized with sodium pentobarbital (Muir et al., 1995).

Posttraumatic hypoperfusion may contribute to the pathophysiology of TBI, as neuropathological evidence of ischemia has been reported in most patients dying after TBI (Graham et al., 1978). In the past, TBI has been reported to result in increases in CBF, termed "luxury perfusion" (Lassen, 1966), or in normal or slightly reduced CBF associated with markedly reduced metabolism (Langfitt and Obrist, 1981). Patients with normal or elevated CBF after severe TBI were significantly more likely to develop intracranial hypertension than patients with CBF levels that corresponded to their reduced cerebral metabolic rates for oxygen (Obrist et al., 1984). Therefore, reduced CBF and posttraumatic ischemia were not thought to play a major role in the pathophysiology of TBI. More recent studies involving CBF measurements within a few hours of injury have shown that nearly one-third of severely injured patients have CBF values below 18 ml/min/100 g and that patients with posttraumatic hypoperfusion have a poorer prognosis than patients with higher CBF levels (Bouma et al., 1992; Miller, 1990). Therefore, evidence that posttraumatic hypoperfusion contributes to secondary injury after TBI suggests that prevention of CBF decreases after injury may be beneficial.

Posttraumatic hypoperfusion is a feature of experimental head injury models. The FP TBI model produces posttraumatic hypoperfusion in cats, pigs, and rats. In cats, CBF does not change significantly in the first hour after injury (DeWitt et al., 1986; McIntosh et al., 1987) but then decreases significantly by 2 h after TBI (McIntosh et al., 1987). In contrast, CBF decreases significantly within 15 min after TBI but returns to baseline within a few hours after TBI in rats and pigs (Yamakami and McIntosh, 1989, 1991; Yuan et al., 1989; Muir et al., 1992; Armstead and Kurth, 1994). Both central and lateral FP TBI produce widespread decreases in CBF in rats. CBF decreases similarly in hemispheres, brain stem, and cerebellum after moderate central FP injury in rats (Yuan et al., 1988). Moderate lateral FP TBI produced significant decreases in CBF in the brain stem, cerebellum, diencephalon, and frontal and parietal cortices on both (i.e., injured and uninjured) sides of the rodent brain (Yamakami and McIntosh, 1989, 1991). Thus, FP TBI models are suited for studying the mechanisms that contribute to posttraumatic hypoperfusion. In addition, although LDF measures perfusion in a small area of the cerebral hemispheres, CBF values in the cerebral hemispheres seem to reflect the global changes in CBF that occur after FP TBI.

Although the mechanisms contributing to posttraumatic changes in CBF are unknown, one possibility is that TBI interferes with the production of or destroys a cerebral vasodilatory substance or substances. A reasonable candidate for the cerebral vasodilatory agent that is

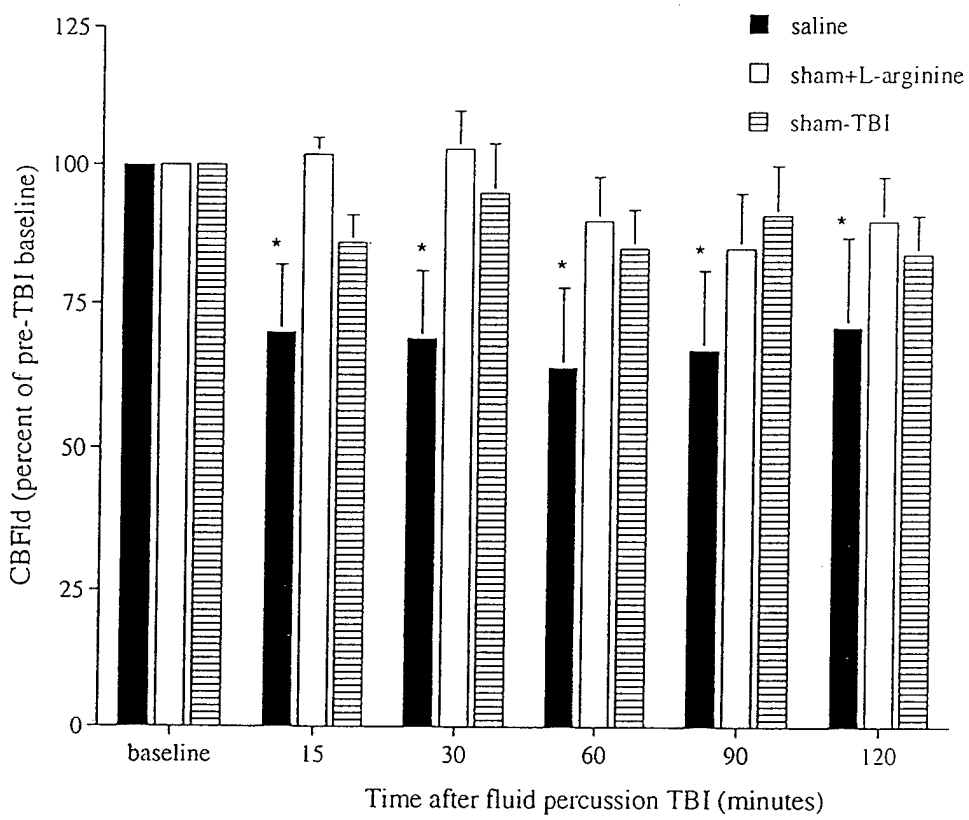

FIG. 2. Laser Doppler cerebral blood flow (CBFld) after traumatic brain injury (TBI) plus saline placebo (saline, $n = 8$) or sham-TBI plus L-arginine (sham + L-arginine, $n = 8$) or sham-TBI with no treatment ($n = 8$). * = significantly ($p < 0.05$) lower than baseline CBF.

affected by TBI is the endothelium-dependent relaxing factor NO. The rodent cerebral circulation exhibits a resting vasodilatory tone mediated by NO (Wang et al., 1992; Pelligrino et al., 1993; Beckman et al., 1991; Tanaka et al., 1991; Faraci, 1991; DeWitt et al., 1992; Kozniewska et al., 1992). Beckman et al. reported that CBF decreased markedly after the infusion of L-nitroarginine hydrochloride (30 mg/kg, i.v.) in halothane-anesthetized rats (Beckman et al., 1991). Tanaka et al., who measured local CBF using iodoantipyrine in awake rats receiving L-nitromonomethyl arginine (L-NMMA, 25 mg/kg, i.v.), found that local CBF was decreased from 20% (substantia nigra) to 33% (hypothalamus) when compared with a saline-treated group (Tanaka et al., 1991). Faraci reported that cerebral arteries and arterioles in barbiturate-anesthetized rats decreased in diameter after topical application of L-NMMA (Faraci, 1991). Kozniewska et al. observed that CBF, measured using intracarotid ^{133}Xenon in rats anesthetized with chloral hydrate, decreased approximately 21% after L-NMMA administration (100 mg/kg, i.v.) (Kozniewska et al., 1992). They also reported that the effects of L-NMMA on CBF could be reversed using L-arginine (300 mg/kg, i.v.) but not D-arginine (300 mg/kg, i.v.). Wang et al., using halothane-anesthetized rats, found that NG-nitro-L-arginine decreased CBF (intracarotid ^{133}Xe) in a dose-dependent manner and that CBF decreases persisted for at least 2 h (Wang et al., 1992). Pelligrino and colleagues reported decreases in regional CBF (radioactive microspheres) of more than 50% after infusion of L-nitroarginine methyl ester (L-NAME, 3 mg/kg/min) in rats anesthetized with 70% N_2O and fentanyl (25 μg/kg/hr) (Pelligrino et al., 1993). Recent autoradiographic studies indicate that L-NAME 30 mg/kg infusion decreases CBF by 17% (parietal cortex) to 49% (hypophysis) across different brain regions in rats (Bonvento et al., 1994). These studies, which were performed using a variety of anesthetic and CBF measurement techniques, consistently demonstrated that the inhibition of NO synthesis decreases CBF and support the hypothesis that there is a resting vasodilatory tone mediated by NO in the rodent cerebral circulation.

Our results, that CBF is preserved after TBI in rats treated with L-arginine but not D-arginine, support the hypothesis that TBI reduces CBF by reducing an NO-mediated cerebral vasodilatory tone. L-arginine has been reported to increase CBF during ischemia (Morikawa et

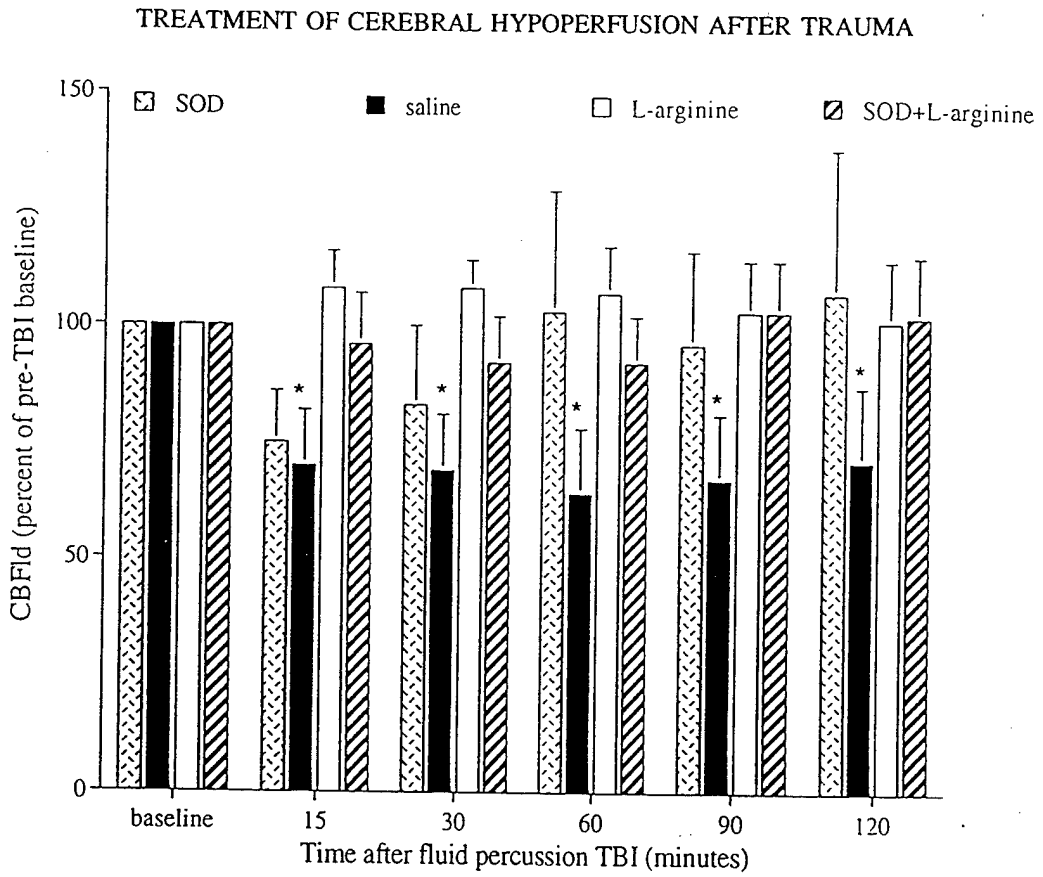

FIG. 3. Laser Doppler cerebral blood flow (CBFld) after traumatic brain injury (TBI) plus superoxide dismutase (SOD) (24,000 U/kg bolus followed by 1,600 U/kg/min infusion for 10 min) (SOD, $n = 8$), TBI plus saline placebo (saline, $n = 8$), TBI plus L-arginine (100 mg/kg) (L-arginine, $n = 10$), or TBI plus SOD and L-arginine (SOD + L-arginine, n = 8). * = significantly ($p < 0.05$) lower than baseline CBF.

al., 1992b; Morikawa et al., 1994) and reduce infarct volume after ischemia (Morikawa et al., 1992b, 1994) in rats. Morikawa et al. also reported that 30 mg/kg and 300 mg/kg L-arginine increased pial arteriolar diameter and that 300 mg/kg L-arginine increased CBF in normal rats (Morikawa et al., 1994). We observed no increase in CBF after the infusion of L-arginine (100 mg/kg) in uninjured rats, perhaps because of the lower dose of L-arginine used in the present study. In addition, CBF responses to L-arginine may be different in rats anesthetized with sodium pentobarbital (Morikawa et al., 1994) than with isoflurane as used in the present study.

If TBI reduces CBF by destroying NO, the mechanism of the effects of TBI on NO remain to be determined. One possibility is that the superoxide anion radical reduces NO levels. Superoxide (O_2^-) anion radical production occurs after FP TBI in cats (Wei et al., 1981; Kontos, 1989; Kontos and Wei, 1992) and rats (Fabian et al., 1995), and reduction of O_2^- activity with SOD or inhibition of O_2^- production with cyclooxygenase inhibitors reduces endothelial damage and restores cerebral vascular reactivity after TBI (Wei et al., 1981; Kontos and Wei, 1992). SOD also restores endothelium-dependent cerebral vasodilatory responses that are markedly attenuated by hemorrhagic hypotension (Szabó et al., 1995). SOD appeared to improve outcome after TBI in Phase II clinical trials (Muizelaar et al., 1993), but more recent results of Phase III clinical trials indicate that SOD may not improve outcome when administered hours after TBI (Young et al., 1996).

NO concentrations, measured using a porphyritic microsensor, decreased during ischemia and then decreased further during reperfusion (Zhang et al., 1995). Superoxide concentrations, measured using a cytochrome C-coated platinum electrode, increase during ischemia and then increase further during reinfusion (Fabian et al., 1995). These observations are consistent with the hypothesis that the superoxide anion radical decreases NO concentrations *in vivo*. Oxygen radical-mediated damage to NO is supported by evidence that antioxidants (mercaptopropionylglycine and catalase) prevent significant decreases in NO_2^- and NO_3^- (stable metabolites of NO) levels that occur during cardiopulmonary bypass in piglets (Morita et al., 1996). In the present study, SOD

TABLE 3. MEAN ARTERIAL PRESSURE (MAP), ARTERIAL BLOOD GASES AND HEMOGLOBIN (Hgb) IN RATS TREATED WITH SALINE (n = 5), L-ARGININE (n = 5) OR D-ARGININE (n = 5) AFTER MODERATE FLUID-PERCUSSION TBI

			Time after TBI (min)					
Variable	*Group*	*Pre-TBI*	*1*	*15*	*30*	*60*	*90*	*120*
MAP	saline	90 ± 12	156 ± 23	91 ± 13	100 ± 15	95 ± 15	98 ± 15	95 ± 15
(mmHg)	L-arg	101 ± 4	121 ± 36	101 ± 3	103 ± 5	101 ± 6	97 ± 8	96 ± 9
	D-arg	108 ± 7	143 ± 25	112 ± 5	116 ± 7	112 ± 3	118 ± 5	116 ± 5
PaO_2	saline	299 ± 5				284 ± 3		
(mmHg)	L-arg	294 ± 6				299 ± 12		
	D-arg	291 ± 16				290 ± 5		
$PaCO_2$	saline	30.5 ± 2.0				38.1 ± 2.9		
(mmHg)	L-arg	38.4 ± 4.9				34.5 ± 1.0		
	D-arg	33.2 ± 2.0				37.3 ± 3.6		
pH	saline	7.48 ± 0.02				7.39 ± 0.02		
	L-arg	7.42 ± 0.04				7.42 ± 0.01		
	D-arg	7.42 ± 0.02				7.40 ± 0.02		
Hgb	saline	12.3 ± 0.6				14.6 ± 0.6		
	L-arg	13.4 ± 0.3				12.9 ± 1.0		
	D-arg	13.1 ± 0.5				12.3 ± 0.4		

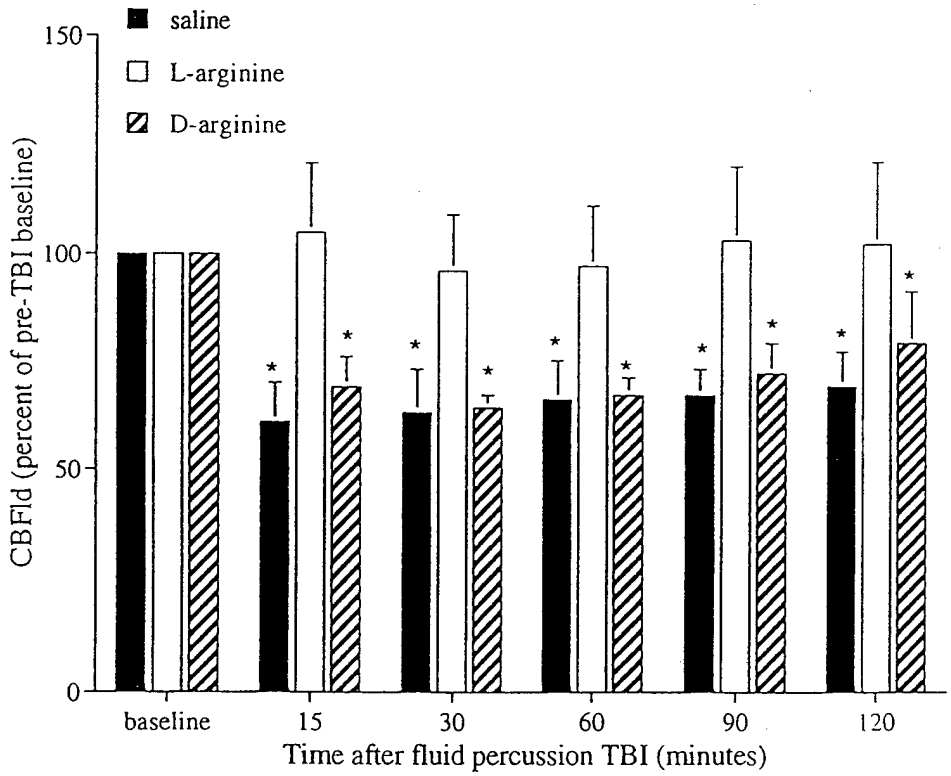

FIG. 4. Laser Doppler cerebral blood flow (CBFld) after TBI plus saline placebo (saline, n = 5), TBI plus L-arginine (100 mg/kg) (L-arginine, n = 5), or TBI plus D-arginine (100 mg/kg) (D-arginine, n = 5). * = significantly ($p < 0.05$) lower than baseline CBF.

did not prevent cerebral hypoperfusion after TBI but restored CBF to baseline after a brief period of hypoperfusion. It is possible that the dose of SOD used in the present studies permitted some transient destruction of NO during the initial burst of O_2^- production that occurs after TBI (Fabian et al., 1995). Superoxide is likely produced during the large increase in prostaglandin production that occurs after TBI in rats (DeWitt et al., 1988). Brain prostaglandin levels increase 20-fold within the first 5 min after moderate FP TBI (DeWitt et al., 1988), perhaps producing levels of O_2^- radicals that were too high to be effectively scavenged by the dose of SOD used in the present studies. The hypothesis that SOD restores CBF by suppressing an initial burst of superoxide production is also consistent with the short plasma half-life of SOD. Native SOD has a plasma half-life of approximately 6 min in rats (Odlind et al., 1988). Therefore, plasma SOD levels would likely have decreased over time after the bolus injection just before TBI. Infusion of SOD immediately after TBI would have slowed the rate of decline in SOD levels, but it is likely that plasma SOD levels decreased markedly after the infusion was stopped. These data suggest that if the initial burst of oxygen radical production can be reduced or if NO substrate levels can be transiently increased, traumatic damage to the cerebral vasculature is minimized and CBF returns to or is maintained at baseline levels.

Superoxide reacts with NO to produce another toxic oxidant, the peroxynitrite anion ($ONOO^-$) (Beckman, 1991), which, when protonated, forms an intermediate species with the reactive properties of nitrogen dioxide and the hydroxyl radical. The relatively high concentrations of SOD and the extremely fast reaction rate of SOD with superoxide normally maintain very low levels of intracellular superoxide anions (Crow and Beckman, 1995). However, the formation of peroxynitrite from superoxide and NO is extremely rapid as well and, if NO is present in sufficient quantities, peroxynitrite formation will predominate (Beckman et al., 1994). What remains to be determined is whether the superoxide radicals produced after TBI are sufficient to overwhelm the available SOD, leading to a shift to peroxynitrite formation (with attendant inactivation of NO). Potentially, excess L-arginine could increase NO concentrations and increase peroxynitrite formation. The concept of conversion of NO to $ONOO^-$ or its destruction by O_2^- through other mechanisms is supported by evidence that SOD markedly increases the stability of NO (Rubanyi and Vanhoutte, 1986). There is clearly a need for further investigations of the role of peroxynitrite in the pathophysiology of TBI.

It is important to note that L-arginine may be acting by a mechanism unrelated to the synthesis of NO. As noted by Morikawa et al., brain intracellular L-arginine concentrations (300–800 μmol/L) are much higher than K_m for NO in the rodent brain (2 μmol/L) (Morikawa et al., 1994; Bredt and Snyder, 1990). Therefore, it is unclear how adding additional substrate to a saturated enzyme system would increase the production of NO enough to overcome decreases in NO that we are suggesting may occur after TBI. It is possible that L-arginine is compartmentalized in certain cells within the neuraxis (Morikawa et al., 1994) and that additional L-arginine would reach cells that have an insufficient supply of substrate. An alternative possibility is that TBI affects NO synthase activity directly and renders L-arginine utilization less effective. Finally, arginine metabolism is complex, and other potentially vasoactive products of arginine metabolism such as polyamines or agmatine may be contributing to the effects of L-arginine on CBF after TBI (Reyes et al., 1994).

These studies demonstrate that posttraumatic hypoperfusion can be prevented by early treatment with the NO precursor, L-arginine, but not by its stereoisomer, D-arginine. Furthermore, they demonstrate that SOD reverses posttraumatic hypoperfusion, suggesting that decreases in CBF that occur immediately after TBI may be due to oxygen radical-mediated destruction of the potent endothelium-derived vasodilatory substance, NO.

ACKNOWLEDGMENTS

The authors thank Christopher Tidwell for technical assistance, Deborah Goodyear for assistance with manuscript preparation, and Kimberly Button for secretarial support. We thank Faith McLellan for editorial review. We are grateful to our colleagues in the Department of Anesthesiology at the University of Texas Medical Branch for their support of the Charles R. Allen Research Laboratories. These studies were supported, in part, by NIH grant 19355 to Dr. DeWitt.

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Traumatic brain injury does not alter cerebral artery contractility

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Bukoski, Richard D., Shi Nan Wang, Ka Bian, and Douglas S. DeWitt. Traumatic brain injury does not alter cerebral artery contractility. *Am. J. Physiol.* 272 (*Heart Circ. Physiol.* 41): H1406–H1411, 1997.—Previous studies have shown that traumatic brain injury (TBI) significantly reduces cerebral blood flow determined in vivo and reduces vascular reactivity in the pial circulation measured with cranial window preparations. We have now tested the hypothesis that TBI induces these changes by impairing intrinsic contractile activity of cerebral arteries. Anesthetized rats underwent moderate (2.2 atm) and severe (3.0 atm) midline fluid percussion TBI or sham injury following which posterior cerebral or middle cerebral arteries were isolated and isometric force generation was measured. Moderate ($n = 5$) and severe ($n = 3$) trauma had no effect on the magnitude of serotonin- or K^+-induced force generation or sensitivity to serotonin in arteries isolated within 10 min of TBI. Functional disruption of the endothelium of posterior cerebral arteries isolated 10 min after moderate trauma or sham injury caused a reduction in the active tension response to serotonin that was similar in both groups. Blockade of cyclooxygenase with 5 μM indomethacin had no effect on serotonin-induced force generated by vessels with moderate trauma or in sham-treated rats. Acetylcholine induced an endothelium-dependent relaxation of posterior and middle cerebral arteries; the magnitude of the response was unaffected by moderate TBI. To determine whether prolonged in situ exposure of vessels to the traumatized cerebral milieu could reveal an alteration in intrinsic contractility, posterior cerebral arteries were isolated 30 min after TBI; again, no differences in the tension or relaxation responses were observed. It is concluded that midline fluid percussion TBI did not affect contraction or relaxation of proximal middle or posterior cerebral arteries in rats.

brain trauma; vascular reactivity; serotonin; endothelium

TRAUMATIC BRAIN INJURY (TBI) in humans is the leading cause of morbidity and mortality in people under the age of 40 yr in Western society (18). Feline (9, 11, 12, 17, 19, 26, 31, 34), rodent (10, 14, 27, 30, 35–37), and porcine (1) models have provided insight into the mechanisms that contribute to the permanent cognitive and motor deficits that result from TBI. Among the early changes observed after trauma are alterations in cerebrovascular function characterized by cerebral hypoperfusion (3, 35–37) and impaired autoregulatory responses to changes in systemic blood pressure and plasma oxygenation (19–21). TBI also decreases compensatory increases in cerebral blood flow (CBF) that normally occur following isovolumic hemodilution (12, 33). Impairment of these regulatory mechanisms is believed to contribute to deficits in neuronal function or to lead to cell death when arterial hypotension occurs after TBI in humans (8, 23).

The mechanisms by which TBI impairs CBF and cerebral vascular reactivity are not yet fully understood. The majority of studies have been performed using in vivo methods, including the measurement of CBF using microspheres (35–37) and in situ analysis of changes in pial artery diameter using the cranial window preparation (17, 34). Among the more consistent results has been the observation of morphological and functional damage to the endothelium (17, 19). It is therefore logical to propose that our mechanistic understanding of the cellular events that occur in cerebral vascular smooth muscle and endothelial cells after trauma would be facilitated by performing ex vivo analyses of vascular tissue taken from animals after TBI. With this in mind, we initiated the studies that are described in this report. Our primary aim was to test the hypothesis that TBI induces changes in cerebral artery contraction and relaxation that can be detected after the vessel is removed from the animal. The results show that central fluid percussion TBI does not induce intrinsic changes in arterial contractility, and they have important implications regarding the mechanisms of TBI-induced changes in CBF regulation.

METHODS

Animal preparation. Male Sprague-Dawley rats weighing 300–350 g were anesthetized with 1.5–2% isoflurane in 70% air-30% O_2 and surgically prepared for midline fluid percussion injury as previously described (14). Briefly, the rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4.8-mm hole was trephined into the skull over the sagittal suture, and a modified Luer-lok syringe hub was placed over the exposed dura and bonded in place with adhesive. We then connected the rats to the trauma device and they were subjected to moderate (2.2 atm) or severe (3.0 atm) TBI. Arterial blood pressure, monitored via a polyethylene catheter in one femoral artery, was recorded using an MP100 computer data-acquisition system (Biopac Systems, Goleta, CA).

After injury or sham injury, the rats were exsanguinated via cardiac puncture. The skin over the calvaria was reflected, and the calvaria was removed by cutting through the lateral walls of the orbit, the temporal bone superior to the zygomatic arch, and the parietal and occipital bones. After the calvaria was removed, the brain was chilled by dousing it with ice-cold saline, after which it was excised, with care taken to avoid stretching cerebral vessels at their site of attachment to the cerebral arterial circle (of Willis). Posterior cerebral arteries, including first and second branches, or middle cerebral arteries were removed and cleaned of loosely associated connective tissue.

Isometric force. Isometric force generation of the isolated vessels was determined using established methods (5). Because the vessels were studied at a standardized length, the

diameter of each segment was not systematically measured. However, cerebral arteries from similar animals studied in our laboratory with a cannulated vessel system have an internal diameter of 75–100 μm when pressurized at 100 cmH_2O. Vessels were mounted on a dual-channel wire myograph and maintained in physiological salt solution (PSS) of the following composition (in mM): 130 NaCl, 4.7 KCl, 1.17 $MgSO_4 \cdot 7\ H_2O$, 5 glucose, 1.50 $CaCl_2$, and 15 $NaHCO_3$. When gassed with a mixture of 95% air-5% CO_2, this solution had a pH of 7.4. After an equilibration period of 30 min at 37°C, the axial length was measured using a filar micrometer eyepiece, and the segments were set to their optimal length for force development by construction of an active length-tension curve using solution containing 100 mM K^+ to stimulate active force generation. The composition of this high-K^+ solution was (in mM): 34.7 NaCl, 100 KCl, 1.17 $MgSO_4 \cdot 7\ H_2O$, 5 glucose, 1.50 $CaCl_2$; and 15 $NaHCO_3$. This solution had a pH of 7.4. We denuded some vessels of the endothelium by stroking a human hair in and out of the lumen of the vessel. To verify the status of the endothelium, each vessel was precontracted with a concentration of serotonin (0.3 μM), which induced ~70% of maximal force, and the relaxation response to acetylcholine was assessed. The absence of a relaxation response to acetylcholine was taken to indicate that the endothelium had been functionally disrupted (22). In some vessels the effect of inhibition of cyclooxygenase was assessed by pretreatment of the vessel with 5 μM indomethacin (22). Concentration-response curves were generated by the cumulative addition of the indicated agonist. All responses were recorded in units of millinewtons and normalized to the axial length of the vessel segment and expressed as active tension in millinewtons per millimeter.

Experimental design. One group of experiments assessed the basic reactivity of posterior cerebral arteries isolated from rats that underwent moderate trauma. These vessels were isolated within 10 min of TBI. A second group of experiments assessed the effect of graded levels of trauma and assessed posterior cerebral arteries after either moderate or severe TBI. In a third set of experiments, the effect of moderate trauma on reactivity of middle cerebral arteries isolated within 10 min after trauma was assessed. A fourth group of experiments assessed the effect of a prolonged posttraumatic incubation period after moderate TBI on the reactivity of subsequently isolated posterior cerebral arteries. In this group, vessels were isolated 30 min after the traumatic event.

Data analysis. Results were analyzed for differences using either Student's *t*-test or analysis of variance with a repeated-measures design (SYSTAT software system). A P value of ≤ 0.05 was assumed to indicate a significant difference.

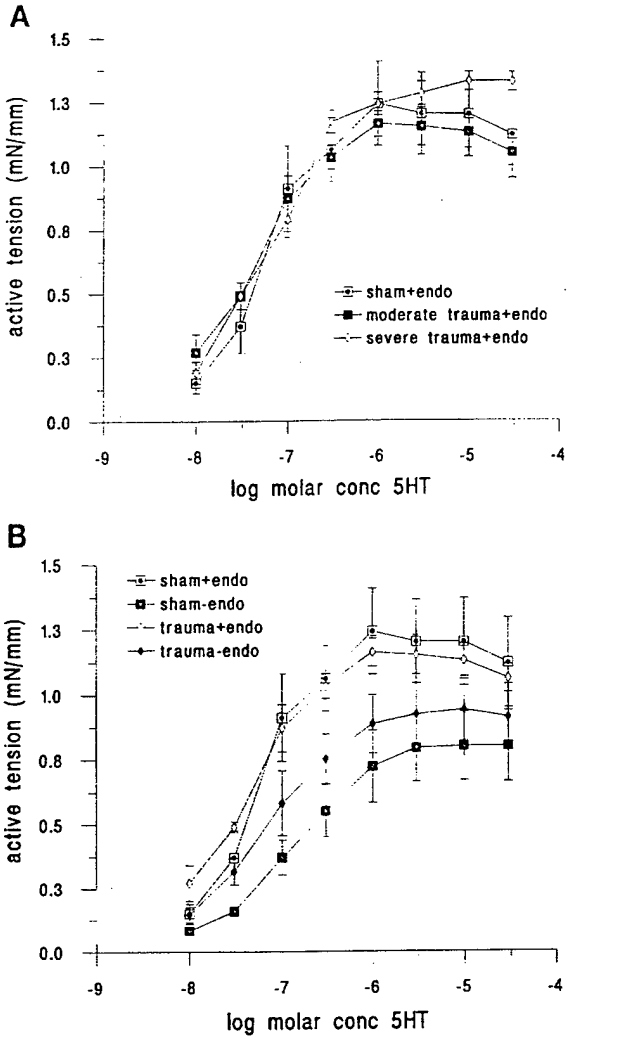

Fig. 1. Effect of trauma on active tension response of posterior cerebral arteries. *A*: effect of moderate or severe trauma on endothelium-intact (+endo) posterior cerebral arteries to cumulative addition of serotonin (5-HT). *B*: effect of endothelial denudation (−endo) on the response to 5-HT of vessels from moderately trauma- and sham-injured animals. Values are means ± SE; $n = 5$ for each group. No significant differences were observed.

RESULTS

In initial experiments, we examined the force responses of posterior cerebral arteries isolated from rats within 10 min of moderate trauma. All vessels were set to their optimal length for force generation. No differences in the active tension (force normalized to axial length of the vessel segment) responses to serotonin (Fig. 1*A*) or to a maximal challenge with 100 mM K^+ (Table 1) were observed. To test the hypothesis that endothelium-independent reactivity was altered, responses were also determined in vessels denuded of endothelium. Endothelial denudation significantly attenuated force generation in vessels from both the trauma- and sham-injured groups, but differences between these groups were not detected (Fig. 1*B*). Vessel

Table 1. *Effect of trauma on tension response to 100 mM K^+*

Vessel	Sham Trauma	Moderate Trauma	Severe Trauma
Posterior cerebral artery (10 min post)	1.77 ± 0.2 (6)	2.00 ± 0.18 (9)	2.19 ± 0.05 (5)
Posterior cerebral artery (30 min post)	2.28 ± 0.37 (5)	2.3 ± 0.11 (3)	
Middle cerebral artery (10 min post)	1.47 ± 0.34 (3)	1.78 ± 0.33 (3)	

Values are means ± SE of active tension response to 100 mM K^+ in mN/mm. Number of separate observations is indicated in parentheses. Post, posttrauma. No differences in the levels of trauma within vessel groups were detected.

Table 2. *Effect of indomethacin on tension response to serotonin before and after moderate trauma*

Vessel/Trauma	Control	Control + Indo	Trauma	Trauma + Indo
Posterior cerebral artery (10 min post)	1.24 ± 0.16 (5)	1.15 ± 0.13 (5)	1.16 ± 0.05 (5)	1.19 ± 0.06 (5)
Middle cerebral artery (10 min post)	0.91 ± 0.24 (3)	0.87 ± 0.22 (3)	1.15 ± 0.13 (3)	1.3 ± 0.22 (3)
Post cerebral artery (30 min post)	1.8 ± 0.11 (4)	1.72 ± 0.22 (4)	1.61 ± 0.14 (6)	1.66 ± 0.13 (6)

Values are means ± SE of the active tension response to K^+ in mN/mm. Number of separate observations is indicated in parentheses. Indo, indomethacin; post, post moderate trauma. No effect of indomethacin was detected in any of the groups.

segments were also studied after inhibition of cyclooxygenase by pretreatment of the segments with 5 μM indomethacin to test the hypothesis that vasoactive prostanoid production was altered. Pretreatment with indomethacin had no effect on the active tension response of either the sham-injured or moderately trauma-injured animals (Table 2).

The relaxation response of these posterior cerebral arteries to acetylcholine after precontraction with serotonin was assessed. As shown in Fig. 2, acetylcholine caused a dose-dependent relaxation of precontracted posterior cerebral arteries that was not different between the sham- and trauma-injured animals and was ablated by removal of the endothelium.

Because these results indicated that moderate TBI was without effect on agonist-induced force generation or relaxation, we considered the possibility that a greater level of trauma would be able to induce a persistent change in vascular reactivity. Posterior cerebral arteries were isolated from animals within 10 min of their undergoing severe (3.0 atm) brain trauma. As with the moderately trauma-injured group, the responses of vessels to serotonin in the severely trauma-injured group (Fig. 1*A*) or to K^+ (Table 1) were not different from those in the sham-injured group.

Because posterior cerebral arteries did not show significant alterations in reactivity after moderate or severe TBI, we considered the possibility that regional differences in reactivity may be induced by TBI and assessed the effect of moderate trauma on the reactivity of middle cerebral arteries. Vessels were again isolated within 10 min of the moderate injury and prepared for measurement of isometric force generation. No differences in either the contractile responses of the vessels to serotonin (Fig. 3*A*) or K^+ (Table 1) or in the relaxation responses to acetylcholine (Fig. 3*B*) were detected ($n = 3$ per group).

As a final consideration, we tested the hypothesis that prolonged in situ exposure of vessels to substances liberated from parenchymal elements or circulating factors induced by trauma might significantly alter the

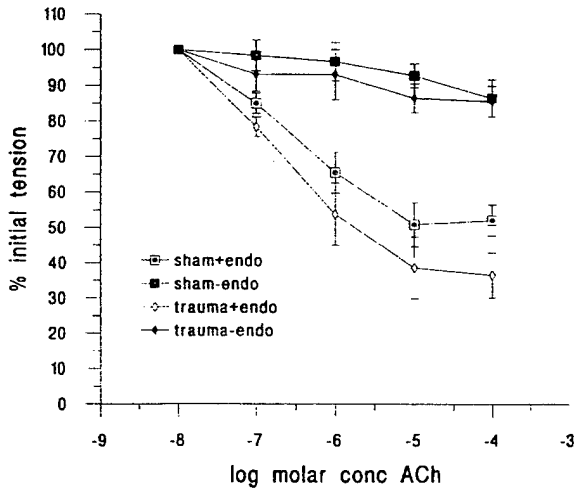

Fig. 2. Effect of moderate trauma on relaxation response of 5-HT-contracted posterior cerebral arteries to cumulative addition of acetylcholine (ACh). Values are means ± SE; $n = 5$ for each group. No significant differences were observed.

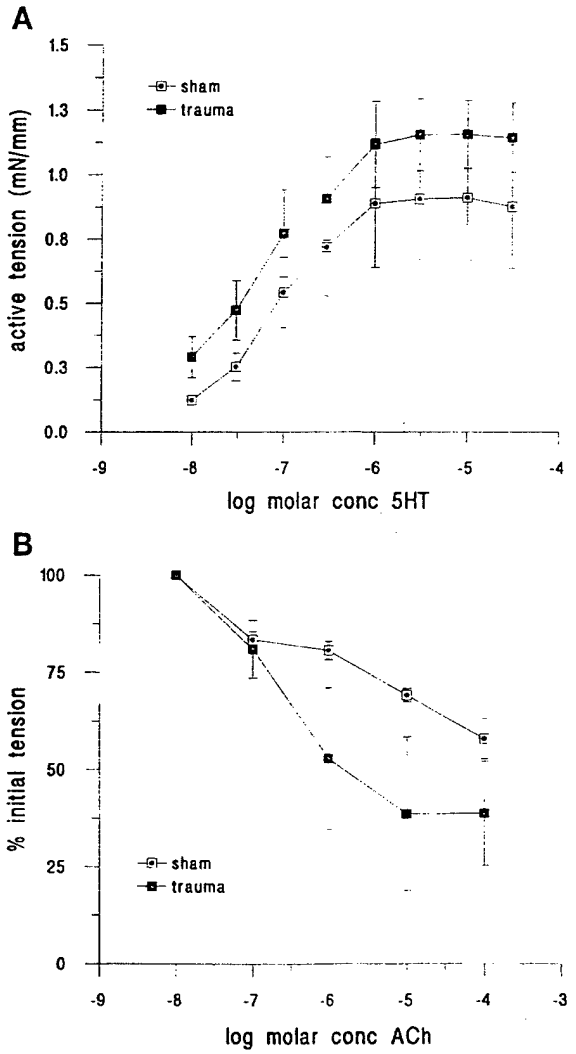

Fig. 3. Effect of moderate trauma on middle cerebral arteries. *A*: effect of moderate trauma on response of endothelium-intact middle cerebral arteries to cumulative addition of 5-HT. *B*: effect of moderate trauma on relaxation response of 5-HT-contracted middle cerebral arteries to cumulative addition of ACh. Values are means ± SE; $n = 3$ for each group. No significant differences were detected.

intrinsic contractility of cerebral arteries. In these experiments, moderate brain trauma was performed, and the animal was maintained in the experimental apparatus for a 30-min period, following which posterior cerebral arteries were isolated and prepared for analysis of isometric force generation. As with the previous experiments, moderate trauma with prolonged incubation had no effect on the contractile responses of vessels to serotonin (Fig. 4*A*) or K^+ (Table 1) or on the relaxation response to acetylcholine (Fig. 4*B*).

DISCUSSION

On the basis of previous reports that both vasoconstrictor (1, 13, 15, 19, 28, 34) and vasodilator (9, 11, 17, 19–21, 34) responses of the cerebral circulation are impaired by TBI, we tested the hypothesis that TBI induces changes in cerebral artery reactivity that can be detected ex vivo. The major new finding of the present study is that midline fluid percussion TBI does not impair the intrinsic contractile ability of subsequently isolated cerebral arteries. In our initial experiments, the effect of moderate trauma on reactivity of isolated posterior cerebral artery segments was assessed. TBI had no effect on the force response to either serotonin or K^+ (Fig. 1). Moreover, TBI had no effect on the endothelium-dependent dilator response to acetylcholine, although removal of the endothelium significantly impaired the magnitude of force generation (Fig. 2). Therefore, our results indicate that neither endothelium-dependent nor endothelium-independent responses were affected by central fluid percussion injury (FPI).

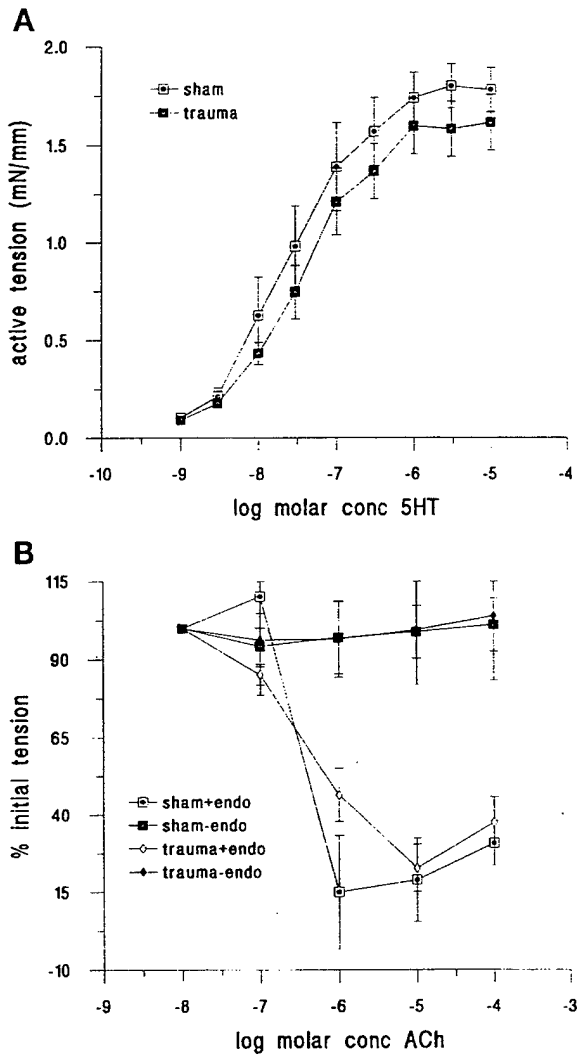

Fig. 4. Effect of posttraumatic in situ incubation on vessel reactivity to 5-HT (*A*) and ACh (*B*). Vessels were isolated 30 min after moderate brain injury and studied with intact endothelia. Values are means ± SE; $n = 3$ for each group. No significant differences were observed.

Regional differences in the degree of impairment of vasodilatory responses to hemorrhagic hypotension can occur after moderate TBI. For example, anterior brain regions in cats exhibit more severe impairment of autoregulation than regions such as the occipital lobes and brain stem (11). We therefore tested the hypothesis that responses in the middle cerebral artery, which supplies more anterior brain regions, may be affected. Our results showed, however, that neither vasoconstrictor responses to serotonin nor the vasodilator responses to acetylcholine are altered by moderate TBI (Fig. 3). Thus regional differences in the arterial responses after moderate, central FPI were not detected in the ex vivo analysis.

FPI has been studied using midline (14), parasagittal (13), and lateral (27) placement of the craniotomy for attachment of the head-injury device. Schmidt and Grady (32) compared blood-brain barrier damage (horseradish peroxidase extravasation) in the three models but did not examine vascular structural damage or cerebral vascular reactivity. Dietrich et al. (13) reported endothelial discontinuities and constricted arterioles in subcortical white matter, hippocampus, and lateral thalamus, but comparable detailed light and electron-microscopic histological analyses have not been performed after midline or lateral FPI. Despite the absence of histological assessments of the effects of midline FPI, other evidence suggests that traumatic effects on CBF and cerebral vascular reactivity are similar after both midline and lateral FPI. CBF decreases similarly in the hemispheres, brain stem, and cerebellum after moderate central FPI in rats (37). Moderate lateral FPI produced significant decreases in CBF in the brain stem, cerebellum, diencephalon, and frontal and parietal cortices on both (i.e., injured and uninjured) sides of the rodent brain (35–37). Although regional autoregulation has not been assessed in rats after TBI, moderate central FPI impairs autoregulation in all 46 brain regions studied in cats (10). Therefore, CBF studies suggest that the effects of moderate, central FPI on the cerebral circulation are not confined to the brain stem. However, we cannot exclude the possibility that TBI affected vessels of a different size or location from those studied or the possibility that lateral FPI may impair the agonist-induced vasoconstrictory or vasodilatory responses that were not affected by central FPI.

It is also recognized that cerebral vascular responses are impaired to a greater degree as the level of TBI increases. Vasoconstrictor responses to hypocapnia in the feline pial arteries and arterioles are reduced by moderate (2.7 atm) TBI and were completely abolished by severe TBI (3.4 atm) (35). Moreover, autoregulatory vasodilatory responses to progressive hemorrhagic hypotension in feline pial arteries and arterioles are reduced by mild (1.6 atm) TBI, whereas higher levels of TBI result in cerebral arterial vasoconstriction during hypotension (35). In the present study, severe TBI (3.0 atm) did not reduce vasoconstriction to serotonin or vasodilatory responses to acetylcholine (Fig. 3). Therefore, the level of TBI has no effect on intrinsic contractile properties of cerebral arteries studied ex vivo.

With continuous monitoring of posttraumatic CBF using laser Doppler flowmetry, Muir et al. (30) reported that CBF decreases gradually during the first 20–30 min after TBI in rats. These observations suggest that traumatic injury to the cerebral vasculature may develop over time. To determine whether there is a gradual impairment of cerebral vascular contractile or dilatory responses after TBI, posterior cerebral arteries were isolated 30 min post-TBI, when CBF decreases are the most pronounced (30, 35). No alterations in contractility or relaxing ability were noted (Fig. 4), indicating that if an injury process develops slowly after TBI in vivo it does not affect fundamental responses to applied agonists in pial arteries.

Although these findings do not support the hypothesis that changes in agonist-mediated vascular reactivity contribute to trauma-induced impairment of cerebral blood flow, one caveat needs to be considered: in addition to agonist-induced contraction and relaxation, cerebral arteries have a significant component of myogenic reactivity (6, 29). It is therefore possible that changes in myogenic reactivity are induced by TBI that would not be detected by the protocols we used. This possibility remains to be examined.

Our results suggesting that the intrinsic mechanisms that regulate vascular reactivity of small cerebral arteries are not altered by TBI have several important implications. One is that alterations in reactivity observed in vivo after brain trauma may be the result of the proximity of the cerebral arteries to the underlying parenchyma of the brain. Factors that need be considered include perivascular innervation, which could modulate reactivity, and exposure to vasoactive factors elicited by trauma from brain parenchyma or arriving at the cerebral circulation via the systemic circulation. Perivascular sympathetic fibers contribute to maintenance of autoregulatory vasoconstriction in response to acute hypertension (2, 7) and, in addition to sympathetic fibers, there are myriad types of perivascular nerve fibers whose function are only partly understood (4). Vasoactive factors produced by TBI include prostanoids (12) and other arachidonic acid metabolites (16), oxygen free radicals (24), endogenous opioids (24, 26), and a variety of other agents (25). Our results also suggest that cerebral arteries remain fundamentally intact and reactive and that proper cerebral vascular function could be restored given proper therapeutic intervention to restore the milieu in which the cerebral circulation exists.

The authors acknowledge the expert technical assistance of Kim Miller and the editorial assistance of Faith McLellan and Wilma Frye.

We thank our colleagues in the Departments of Medicine and Anesthesiology for support of the Hypertension and Vascular Research Laboratories and the Charles R. Allen Research Laboratories, respectively.

This work was funded, in part, by a grant from the National Institute of Neurological Disorders and Stroke (NS-19355).

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Received 18 June 1996; accepted in final form 17 September 1996.

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Editorial Comment

A variety of pharmacological approaches have been used to prevent or reduce histopathologic and neurological injury after experimental ischemic[1,2] and traumatic[3,4] brain injury. Manipulation of catecholamine systems with amphetamine or direct intraventricular injections of norepinephrine produces lasting improvements in motor behavior after cortical ablation injury.[5,6] In addition to pharmacological interventions, environmental enrichment strategies, even when applied weeks after injury, improve performance on motor behavioral assessments after experimental focal ischemia.[7,8] Such studies have important implications both for the acute treatment and the subsequent rehabilitation of patients after traumatic brain injury or stroke.

Most studies of cerebral trauma and ischemia have focused on the reduction of injury acutely or, more recently, on the reduction of vulnerability to secondary cerebral injury due to hypotension, hypoxia, or other subsequent systemic challenges. In contrast, the accompanying study by Puurunen et al and those from other laboratories[5-8] represent an interesting focus on later treatment and more prolonged assessments of recovery. In addition, while previous studies involving manipulations of adrenergic neurotransmitters or experience-based strategies have used focal brain injury models, Puurunen and colleagues tested the effects of these manipulations on behavioral and histopathological changes in a model of global cerebral ischemia. Their results indicate that environmental enrichment and labyrinth training were associated with some improved performance at certain times after ischemia. This work is encouraging because it confirms results of previous studies[7,8] performed using a different combination of behavioral assessment methods (ie, beam walking, leg placement, climbing) in a different (focal) model of transient cerebral ischemia. Studies of environmental enrichment have exciting implications for improving long-term recovery after cerebral injuries, but the mechanisms by which environmental changes improve behavioral recovery without altering apparent lesion size are not known. Although environmental enrichment increases synaptic plasticity, the histological analyses of Puurunen et al were not designed to determine whether increases in dendritic numbers or branching occurred. Despite the uncertainty about the mechanisms involved, the accompanying data contribute to an important research area that likely will have a significant impact on therapy and rehabilitation for patients after stroke and traumatic brain injury.

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Traumatic Brain Injury Does Not Affect Nitric Oxide Synthase Activity in Rats

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ABSTRACT

Experimental traumatic brain injury (TBI) damages cerebral vascular endothelium and reduces cerebral blood flow (CBF). The nitric oxide synthase (NOS) substrate, L-arginine, prevents CBF reductions after TBI, but the mechanism is not known. This study examined the possibility that posttraumatic hypoperfusion is due to reductions in the substrate sensitivity of NOS which are overcome by L-arginine. Isoflurane-anesthetized rats were prepared for TBI (midline fluid-percussion, 2.2 atm), sham-TBI, or no surgery (control), and were decapitated. The brains were removed and homogenized or minced for measurements of crude soluble or cell-dependent stimulated NOS activity, respectively. Baseline arterial oxygen, carbon dioxide, pH, or hemoglobin levels did not differ among control, sham, or TBI groups. Total cortical soluble NOS activity in TBI-treated rats was not significantly different from either untreated or sham groups when 0.45 μM or 1.5 μM L-arginine was added. Also, there were no differences in cell-dependent NOS activity among the three groups stimulated by 300 μM N-methyl-D-aspartate, 50 mM K^+, or 10 μM ionomycin. These data suggest that TBI reduces CBF by a mechanism other than altering the substrate specificity or activation of nNOS.

INTRODUCTION

Traumatic brain injury (TBI) results in reduced cerebral blood flow (CBF) in the first few hours after injury (Bouma et al., 1992; Bouma et al., 1991). Although the role of posttraumatic hypoperfusion in pathophysiology is not known, evidence of ischemia in most TBI patients (Graham et al., 1978) suggests that CBF reductions may be important contributors. Early posttraumatic hypoperfusion occurs after experimental TBI (Yuan et al., 1988; Yamakami and McIntosh, 1989; Yamakami and McIntosh, 1991). The causes of significant reductions in CBF after TBI in patients or experimental animals are not known but posttraumatic hypoperfusion may result from impairment or destruction of a cerebral vasodilatory mechanism. The endothelium-dependent relaxing factor, nitric oxide (NO), is one such cerebral vasodilator (Furchgott and Zawadzki, 1980; Ignarro et al., 1987). Evidence that inhibition of NO synthesis decreases CBF (Beckman et al., 1991; Tanaka et al., 1991; DeWitt et al., 1992; Pelligrino et al., 1993) suggests a resting cerebral vasodilatory tone owing to the continuous production of NO. Nitric oxide is a free radical that is inactivated (Rubanyi and Vanhoutte, 1986) or converted to an even more reactive species such as the peroxynitrite anion (Beckman, 1991; Beckman et al., 1994; Crow and Beckman, 1995) by oxygen free radicals such as superoxide.

Superoxide anion radicals are produced by fluid-percussion TBI (Wei et al., 1981; Fabian et al., 1995), perhaps as a byproduct of trauma-induced increases in prostaglandin synthesis (DeWitt et al., 1988). Free radicals contribute to the pathophysiology of TBI because the cyclooxygenase inhibitor indomethacin or the free radical scavenger superoxide dismutase reduces impaired cerebral vascular reactivity and endothelial damage after TBI (Wei et al., 1981). Recent evidence that treatment with L-arginine or superoxide dismutase improves CBF after experimental TBI (DeWitt et al., 1997) supports the hypothesis that radical-mediated inactivation of NO or nitric oxide synthase (NOS) contributes to posttraumatic hypoperfusion. Whether TBI affects NO levels directly or reduces NO by affecting NOS remains unclear. To determine whether NOS activity is altered by brain trauma, the conversion of L-arginine to citrulline was measured in rats subjected to moderate central fluid-percussion TBI.

MATERIALS AND METHODS

Surgical Preparation

All experimental protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas Medical Branch. Male Sprague-Dawley rats weighing 350-400 g were anesthetized with isoflurane in an anesthetic

chamber, intubated, and mechanically ventilated with 1.5-2.0% isoflurane in O_2:room air (70:30) using a volume ventilator (EDCO Scientific, Chapel Hill, NC). Polyethylene cannulae were placed a femoral artery and vein for drug infusion and arterial pressure monitoring, respectively. Rectal temperature was monitored using a telethermometer (Yellow Springs Instruments, Yellow Springs, OH) and maintained using a thermostatically controlled water blanket (Gaymar, Orchard Park, NY). Rats were prepared for midline fluid-percussion TBI as previously described (Dixon et al., 1987). Briefly, the rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4-mm hole was trephined into the skull over the sagittal suture approximately midway between lambda and bregma and a modified Luer-Lok syringe hub was placed over the exposed dura and bonded in place with cyanoacrylic adhesive and covered with dental acrylic. Isoflurane was decreased to 1.5%; the rats were connected to the trauma device and subjected to moderate (2.2 atm) TBI. The animals in the sham group were subject to the same surgical procedure as the TBI group with the exception of the actual insult. Brains were harvested 30 min after surgery (control group), TBI, or sham-TBI and prepared using the crude enzyme assay or the NOS mince assay described below. All assays were performed blinded as to experimental group.

NOS Crude Enzyme Assay

Measurements of soluble NOS activity in crude enzyme preparations based on methods of Bredt and Snyder (Bredt and Snyder, 1989) were made by obtaining and homogenizing the cerebral cortex in three volumes buffer containing 0.32 M sucrose, 20 mM HEPES, 0.5 mM EDTA, and 1 mM dithiothretol. This homogenate was centrifuged for 5 min at 3000 × g. The supernatant was spun at 20,000 × g for 15 min. The supernatant from the second spin was passed over a Dowex AG50WX-8 ion exchange column to remove the endogenous arginine. Activity of the soluble NOS enzyme was monitored by adding Ca^{2+} (0.9 mM), NADPH (10 mM), and [^{3}H]arginine (50 nM) to the homogenate and measuring the levels of [^{3}H]citrulline produced in a 20-min incubation at room temperature. In a group of rats, the NOS inhibitors 3-bromo-7-nitroindazole or s-methyl-tho thiocitrulline were added during this incubation period. The [^{3}H]arginine was separated from the [^{3}H]citrulline by ion exchange chromatography as described below. These data are presented as amount of [^{3}H]citrulline divided by the amount of [^{3}H]arginine + [^{3}H]citrulline × 100 / mg of protein.

NOS Mince Assay

Cell-dependent, stimulated NOS activity in cortical minces was also determined by a modification of the method of Bredt and Snyder (Bredt and Snyder,

1989). The animals were killed by decapitation and their brains removed and placed into ice-cold, oxygenated (95% O_2, 5% CO_2) modified Krebs bicarbonate buffer containing 0.3 mM $CaCl_2$, 118 mM NaCl, 3.3 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 14.2 mM glucose, and 1.2 mM $MgSO_4$. The cortex was dissected and cross-chopped at 450 μm on a McIlwain tissue chopper. The minces were transferred to fresh ice-cold buffer and incubated on ice for 15 min. The buffer was changed and the minces were incubated at 37°C for 1h with two additional buffer changes.

The minces were then transferred to a conical bottom tube on ice for gravity packing. Minivials were prepared that contained 30-µl aliquots of the appropriate drug. Freshly oxygenated buffer (270 µl) containing 30 nM L-[^{3}H]arginine (Amersham) was added. Fifty µl of the gravity-packed slices were added in rapid succession to the minivials and incubated for 5 min. The reaction was stopped with 700 µl of an ice-cold solution of 4 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM unlabeled L-arginine dissolved in buffer. The minivials were then centrifuged at 1000 × g and the supernatant decanted. The pellet was sonicated in 1 ml of 1 M trichloracetic acid (TCA). The vials were then spun at 12,000 × g and an aliquot of the supernatant was collected and extracted three times with two volumes of ethyl ether to remove the TCA. The ether was allowed to evaporate overnight and a 0.5-ml aliquot of each sample was passed through 2 ml of a sodium equilibrated ion

exchange resin (Dowex AG50W-X8) to separate the L-[^{3}H]citrulline formed from L-[^{3}H]arginine. L-[^{3}H]Citrulline was eluted in 4 ml of HEPES and combined with the sample effluent. L-[^{3}H]Arginine was eluted in 6 ml of HEPES at pH 12. This procedure eluted 98% of the total added radioactivity. Cell-dependent NOS activity is presented as [^{3}H]citrulline/[^{3}H]arginine + [^{3}H]citrulline × 100 minus a boiled tissue blank value divided by percent conversion of the same day untreated control.

Experimental Design

All rats were prepared for sham injury or fluid-percussion TBI as described above. Control rats were decapitated without surgical preparation. To measure total baseline soluble NOS activity, control rats (n=10) or rats subjected to sham injury (n = 5) or moderate (2.2 atm), central, fluid percussion TBI (n=5) were decapitated approximately 30 min after injury or sham-injury. Brain were removed and prepared for crude enzyme assessments of total soluble NOS activity as described above.

To measure cell-dependent NOS activity, control rats (n=9) or rats subjected to sham-injury (n = 5) or moderate (2.2 atm) central fluid-percussion TBI (n = 5) were decapitated approximately 30 min after injury or sham injury. Brains were removed and mince preparations were prepared for assessment of NOS activity in response to stimulation with 300 μM NMDA, 50 mM potassium chloride, or 10 μM

ionomycin.

In both studies, statistical differences were determined by analysis of variance followed by Dunnett's test where appropriate. A p value of < 0.05 was considered significant.

RESULTS

All values in the text, tables and figures are means ± standard error of the mean. Baseline mean arterial pressure (MAP) in the rats prepared for measurement of cell-dependent NOS activity in mince preparations was significantly higher than baseline MAP in the rats prepared for the crude soluble enzyme assay; however, there were no significant differences in MAP between the sham-injured and the injured groups. There were no differences among the groups in baseline (pretrauma) arterial oxygen, carbon dioxide, pH, or hemoglobin levels (Table). Levels of fluid-percussion TBI (2.2 atm, 22 msec) and MAP during transient arterial hypertension in response to TBI were the same in all groups (Table).

Total cortical soluble NOS activity was measured in control (n=10), sham-injured (n = 5), or moderate TBI (n = 5) rats with either 0.45 μM or 1.5 μM added exogenous arginine (Figure 1). There were no significant differences in soluble NOS activity between control, sham, or TBI groups at either concentration of added

arginine. L-nitro-arginine methyl ester (L-NAME) blocked 97% of NOS activity when used to perfuse the brain during the surgery or when added during the enzyme assay. 3-bromo-7-nitroindazole and S-methyl-thiocitrulline, more specific inhibitors of the neuronal isoform of NOS, blocked 96% and 99% of NOS activity, respectively, when used during the enzyme assay (unpublished observations).

Baseline and stimulated cell-dependent NOS activity were measured in a mince preparation in control rats (n = 9) and in rats after sham injury (n = 5) or moderate fluid-percussion TBI (n = 5). There were no significant differences among the groups at baseline or after stimulation with NMDA, ionomycin, or potassium chloride (Figure 2).

DISCUSSION

These studies demonstrated that moderate central fluid-percussion TBI did not significantly reduce basal soluble NOS activity or cell-dependent NOS activity in response to stimulation with the glutamate receptor agonist NMDA, to depolarization by KCl, or to a calcium ionophore (ionomycin).

Reduced CBF after TBI occurs in humans (Bouma et al., 1991; Kobayashi et al., 1991) as well as in experimental animals (Yamakami and McIntosh, 1989; Yamakami and McIntosh, 1991; Yuan et al., 1988; Armstead and Kurth, 1994). One

possible mechanism by which this could occur is a reduction in the amount of NO, the major endothelium-derived relaxing factor (Ignarro et al., 1987), which is generated and released. Because the amount of NO that is released is directly proportional to the activity of NOS, it is possible that reduction of NOS activity would lead to a reduction in NO-mediated vasodilation. Reduction of NOS activity could be the result of enzyme destruction or an alteration in substrate or cofactor dependence after injury. Studies showing increased CBF after infusion of L-arginine, the NOS substrate, after TBI (DeWitt et al., 1997) suggest that changes in substrate availability may be the mechanism of reduced CBF. Our experiments failed to show any significant changes in total soluble NOS activity at either concentration of added substrate, suggesting that neither the amount of NOS nor its affinity for arginine was affected by moderate TBI. Additionally, it is possible that NOS cofactor dependence or other changes related to calcium-mediated activation could lead to decreased NO synthesis. In this case, there would be a smaller response to drugs that stimulate NOS. We have previously shown that NMDA, K^+, and a calcium ionophore, ionomycin, each stimulate NOS activity by different calcium-dependent mechanisms (Alagarsamy et al., 1994). However, TBI had no effect on either basal or stimulated NOS activity in the cortex. These data suggest that TBI does not affect the ability of NOS to synthesize NO under a variety of conditions.

An alternate explanation is that the during preparation of the tissue, either during the surgery itself or during the preparation of tissue for the enzymatic assay, there was some artifact-induced reduction in baseline NOS activity. In this case, a further reduction in NOS activity due to TBI may not have been detected. It is possible that the isoflurane used during the surgical procedure may have significantly inhibited NOS activity (Terasako et al., 1994; Tobin et al., 1994). However, because the anesthetic was turned off 30 min before the rats were decapitated, the residual amount present in the *in vitro* preparations would have been minimal (Berg-Johnsen and Langmoen, 1987). Additionally, the levels of enzyme activity in both the crude enzyme preparation and the mince preparation were similar in the untreated animals and the sham surgery controls, suggesting that the isoflurane did not significantly inhibit NOS in our preparation.

Although previous studies suggest that changes in substrate availability are a likely mechanism by which NOS activity might be altered, it is possible that sensitivity to the other cofactors necessary for NOS activation may be changed. Also, although the affinity of NOS for arginine may not be affected, it is possible that the availability of arginine is changed as a result of TBI. It is possible that TBI causes a reduction in the transport of arginine into the cells, thereby making arginine a limiting factor. Therefore, adding exogenous arginine may increase CBF after TBI

(DeWitt et al., 1997) by helping to overcome reductions in transport efficiency. However, our experiments suggest that this may not be the case since, in the mince preparation, there were no differences in the total [^{3}H]arginine uptake in the various groups (data not shown).

Another possibility is that there are significant changes in NOS activity that are localized to areas immediately surrounding the injury site. Because we used the total cortex for our analyses, it is possible that any effect that occurred in those areas most affected was diluted by the surrounding less injured tissue. Regional effects of TBI on NOS activity and therefore on CBF seem unlikely as hypoperfusion occurs throughout the cerebral hemispheres after fluid-percussion TBI in rats (Yamakami and McIntosh, 1989; Yamakami and McIntosh, 1991; Yuan et al., 1988).

Although our data suggest that TBI does not affect total or stimulated NOS activity, it is still possible that there may be changes in NOS after TBI that were not detected. Although most NOS activity was blocked by the specific nNOS inhibitor, there was some residual activity. It is possible that this residual activity represented the pool of NOS that is sensitive to TBI. However, experiments attempting to identify changes in the residual pool were unsuccessful as the remaining activity was below the sensitivity limits of our assay.

These studies demonstrated that moderate TBI does not affect basal or

stimulated NOS activity. Although moderate TBI had no measurable effect on NOS activity, it is possible that a more severe TBI could have produced significant changes. Our present observations that NOS activity is unaffected by TBI, coupled with previous evidence that L-arginine improves CBF after TBI (DeWitt et al., 1997), suggest that TBI decreases NO levels by destroying NO directly, rather than by affecting NO production. This hypothesis is supported by evidence that TBI produces the superoxide anion radical (Wei et al., 1981; Fabian et al., 1995), which inactivates NO (Rubanyi and Vanhoutte, 1986), or rapidly converts it to the powerful oxidant, peroxynitrite (Beckman, 1991; Beckman et al., 1994). Further studies involving direct measurements of NO levels after TBI are required to determine the effects of TBI on NO within the central nervous system and the cerebral vasculature.

Table. Mean arterial blood pressure (MAP, mmHg) and arterial blood gas values (mmHg) and hemoglobin (Hgb, g/dl) levels in rats prepared for measurement of baseline or stimulated NOS activity after moderate fluid percussion TBI or sham TBI.

Experiment	Group	MAP	MAP	Arterial Blood			
		baseline	peak	pH	pO_2	pCO_2	Hgb
Crude Enzyme Preparation							
	Sham	104.2±1.4	---	7.38±0.01	300±16	40±1	12.3±0.2
	TBI	103.5±1.7	140.7±5.7	7.37±0.01	303±23	38±1	12.8±0.3
Mince Preparation							
	Sham	121.3±2.0	---	7.40±0.01	311±18	38±1	12.3±0.2
	TBI	126.5±2.0	155.8±7.6	7.40±0.01	303±13	37±1	13.2±0.2

Data are means ±SEM.

ACKNOWLEDGEMENTS

The authors thank Christopher Tidwell for technical expertise, Faith McLellan for editorial assistance, and Deborah Robb and Lori Spurger for help with organizing and typing this manuscript. We are grateful to Donald S. Prough, M.D., and our colleagues in the Department of Anesthesiology at the University of Texas Medical Branch for their support of the Charles R. Allen Research Laboratories. This work was supported, in part, by NS19355 (NIH/NINDS) and DAMD17-97-1-7008 (Department of the Army).

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